THE
BIOLOGICAL BULLETIN

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Vol. 86, No. 1 February, 1944

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THE OCCURRENCE OF A DIABETOGENIC FACTOR IN THE EYESTALKS OF CRUSTACEANS *

A. A. ABRAMOWITZ, F. L. HISAW AND D. N. PAPANDREA

(Woods Hole Oceanographic Institution, Woods Hole, Massachusetts)

During an investigation of an endocrine influence on carbohydrate metabolism of crustaceans, it was found that aqueous extracts of the eyestalks produced within a very short time an intense hyperglycemia in the blue crab, *Callinectes sapidus*. This observation had been made several weeks previously by Mr. J. Armstrong [1] in the crayfish, and it now appears that a diabetogenic factor may exist in the eyestalks of the decapod crustaceans at least, and that it may be an agent in the normal regulation of sugar metabolism in this group of animals.

Of the common large decapods of the Woods Hole area the lobster has been found to be a very suitable form for such an investigation, but owing to legal difficulties in obtaining this species the blue crab was used. Blood was obtained by heart punctures made through a small hole drilled in the carapace, and subsequently analyzed by the method of Miller and Van Slyke.[2] The use of an anticoagulant was found unnecessary. The blood sugar values represent the total reducing substances, and for the present no distinction between fermentable and nonfermentable sugar was made. The amount of blood withdrawn for each analysis was 0.5 cc.

The blood sugar values of crabs freshly brought into the laboratory vary considerably. As an example, the individual values of seven crabs were 16, 112, 52, 18, 60, 27 and 55 mgs. per cent. This considerable variation is due to the handling of the animals, for it disappears when the crabs are segregated into separate containers and left undisturbed. After 5 days of isolation and starvation, the blood sugar values fell gradually, so that on the fifth day the individual readings of the five crabs showing the highest of the above values were 25, 24, 22, 24 and 21 mgs. per cent. Excess handling of the animals or other forms of excitement tends to induce hyperglycemia even after segregation and fasting. Consequently, blood samples were obtained as rapidly as possible and with a minimum of disturbance to the animals. When extracts were to be tested for glycemic effects, the animals

* Contributions from the Woods Hole Oceanographic Institution, No. 329.

[1] Personal communication.

[2] Miller, B. F. and D. D. Van Slyke, 1936. *Jour. Biol. Chem.*, **114**: 583.

were always separated into individual containers and starved for at least 5 days previously.

1. The Effect of a Single Injection of Eyestalk Extract

Twenty-one crabs were prepared as previously described. The average blood sugar value for this group was 20 mgs. per cent. The animals were divided into four groups of five animals each, so chosen that the average value of each group was 20 ± 1–2 mgs. per cent. Into each animal, 0.2 cc. of a sea-water soluble fraction of the eyestalks of *Uca pugilator* was injected, this volume being equivalent to one Uca eyestalk. Blood samples were taken from the first group one hour following injection, from the second group 2 hours after injection of this group, from the third at 3 hours after injection, from the fourth 4 hours after injection, and

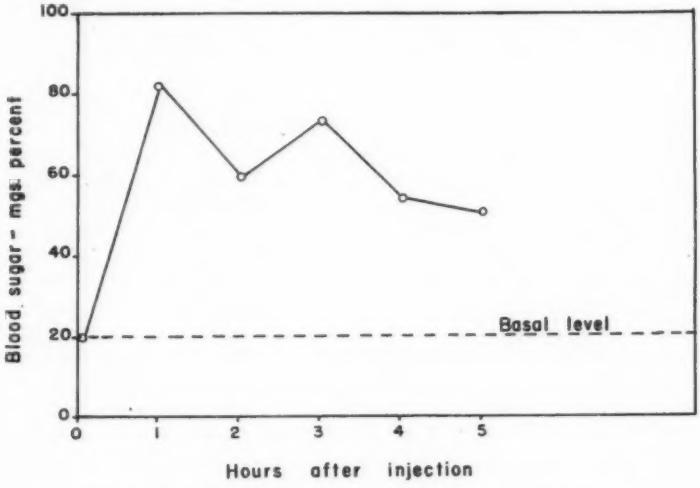

Figure 1. Curve showing the effect of a single injection of eyestalk extract (Uca) on the resting blood sugar level of the blue crab.

again from the first group 5 hours after the injection of this group. The results of this experiment are shown in Figure 1. The diabetogenic effect of the eyestalk extract is not due to reducing substances contained in the extract for, in the above experiment, the amount of extract injected into each animal contained only 1.3 mgs. per cent reducing substances calculated as glucose.

2. The Effect of Dilution on the Diabetogenic Action of Eyestalk Extracts

Eyestalks of *Uca pugilator* were ground thoroughly with a small amount of sea-water, the soluble portion decanted, and the residue re-extracted twice in the same fashion. The combined soluble portions were centrifuged, and from the super-

natant solution a series of dilutions ranging from 5 E.S.[3]/cc. of solution to 0.005 E.S./cc. solution was made. The amount of material injected into the test animals prepared as described above, was in all cases 0.2 cc. Blood samples were withdrawn from each specimen one hour following the injection. The results of this experiment are shown in Table I. It is evident that a rough agreement between

TABLE I

The effect of dilution on diabetogenic activity of the extracts

Number of animals injected	Dosage of extract	Blood sugar values—mgs. %
5	1.0 E.S.	82.4 ± 5.1
4	0.5 E.S.	59.5 ± 10.3
8	0.1 E.S.	49.5 ± 5.5
5	0.05 E.S.	47.0 ± 6.2
7	0.01 E.S.	46.4 ± 11.1
10	0.001 E.S.	41.6 ± 3.9
12	0.2 cc. sea water	24.5 ± 2.3
18	uninjected controls	20.0 ± 1.6

dosage and the resulting hyperglycemia exists, the greatest dose producing the greatest increment. However, there is little difference in the values obtained among the doses ranging from 0.1 E.S. to 0.001 E.S. The greatest response produced was a four-fold increase (1.0 E.S.) but it should be remarked that five- and six-fold increments in blood sugar values have been obtained with extracts of the eyestalks of Callinectes in the same, or even smaller doses. Injection of sea-water was without effect, the slightly higher value (24.5 mgs. per cent) for this group over the uninjected controls (20.0 mgs. per cent) being statistically insignificant. The extract was active in the lowest dosage given (0.001 E.S.) since the value obtained (41.6 mgs. per cent) represents a significant difference over either the uninjected or the sea-water injected control groups. However, some of the readings obtained with higher doses are not significant, this being due to the large standard deviation.

3. The Effect of Boiling on the Diabetogenic Effects of the Extract

Concentrated aqueous extracts of Uca eyestalks were divided into halves, one of which was placed in a water bath at 100° C. for several minutes, and the other left untreated. After heat coagulation, the boiled extract was centrifuged and the supernatant fluid diluted to the original volume before boiling. Both boiled and unboiled extracts were greatly diluted with sea-water so that each animal was injected with 0.2 cc. of a dilution containing 0.5 E.S./cc., or 0.1 E.S. total dose per animal. Blood samples were taken as usual one hour following injection. No difference was found in the activity of the boiled and unboiled extracts (Table II).

4. Localization of the Diabetogenic Activity of the Eyestalk

Four eyestalks of the blue crab were opened by a longitudinal incision, and the sinus gland of Hanström dissected out.[4] The glands were macerated in 4 cc. of

[3] The letters E.S. are used as an abbreviation of eyestalk.

[4] The position of the sinus gland was kindly demonstrated by Dr. F. A. Brown, Jr.

TABLE II

The heat stability of the diabetogenic factor

Dosage	Number of animals injected	Average blood sugar values—mgs. per cent	
		Boiled	Unboiled
0.1 E.S.	4, 4	56	53
0.1 E.S.	6, 6	61	54

sea-water and centrifuged. 0.25 cc. of the extract was injected into each of four animals, the amount being equivalent to one-fourth of a sinus gland. The eyestalks from which the sinus glands were removed were also macerated with 4 cc. of sea-water, centrifuged, and 0.25 cc. of the extract injected into each of four test specimens. One hour after injection, the average blood sugar of the group receiving the sinus gland extract was 83 mgs. per cent, and that receiving the eyestalk (minus sinus gland) extract was 30 mgs. per cent.

5. Effects of Extracts of other Tissues

Saline extracts of the hepato-pancreas of the blue crab were without effect on the resting blood sugar level. Similar extracts injected into specimens made hyperglycemic (100–120 mgs. per cent) by various means produced variable results; in two cases, a sharp fall was obtained while in the majority of cases insignificant changes were observed.

6. The Effect of Eyestalk Extirpation

Twenty-four crabs were isolated and starved for 3 days, after which time the eyestalks were surgically removed from 12 animals, the other 12 remaining as controls. The animals were then starved for another 7 days during which three sets of analyses were obtained from both normal and operated groups, on the second, fifth and seventh days following operation. The results are shown in Table III.

TABLE III

Animals	Blood sugar—mgs. per cent		
Days after operation	2	5	7
Normal (12)	22.6 ± 1.5	25.2 ± 4.5	22 ± 1.6
Operated (12)	24.4 ± 1.6	36.7 ± 4.6	40.3 ± 10.2

The blood sugar of the operated specimens tends to increase following the removal of the diabetogenic factor of the eyestalks, but the differences between the two sets of animals are not significant due to the large standard deviation. The results, however, seem paradoxical to those obtained from injection experiments, but it must be remembered that the operative injury may be sufficient to produce these high and

irregular values and thus to mask any possible hypoglycemia resulting from the removal of the eyestalk factor. This experiment should therefore be repeated with animals from which the sinus gland only is removed.

Summary

A powerful diabetogenic factor has been found in aqueous extracts of the eyestalks of crustaceans. The activity of the extracts is interspecific, heat stable, and effective over a wide dilution range. The sinus gland of Hanström appears to contain most of the diabetogenic factor.

STUDIES ON THE POSTEMBRYONIC DEVELOPMENT OF HYALELLA AZTECA (SAUSSURE)[1]

SISTER FRANCIS SOLANO GEISLER, S. S. J.

(Catholic University of America, Washington, D. C.)

Hyalella azteca is a common and widely distributed fresh-water amphipod crustacean. The genus Hyalella is the only one of the family Talitridae occurring in the fresh waters of America. The species *H. azteca* described by Saussure in 1858 from Vera Cruz, Mexico, where it was originally found among the ruins of the Aztec Indians, is widespread in North and South America, and has been cited in the literature more often under the synonyms *Hyalella knickerbockeri* (Bate) or *H. dentata* Smith.

Holmes (1902, 1903) made observations on *H. dentata* Smith to determine the mode of sex recognition, food habits, thigmotaxis, phototaxis, and reaction to pressure. Weckel (1907) discussed the synonymy of this species and gave a minute description of its characteristic external features. Embody (1911), interested in propagating amphipods as food for fishes, made a study of the distribution, food, and reproductive capacity of four common fresh-water amphipods, including *Hyalella knickerbockeri*. He states that this amphipod breeds for 152 days during the warmer months of the year and averages about 18 eggs (15 times in 152 days) per brood.

Jackson (1912) investigated the distribution and habits of this species, also its color, size, moulting, effects of starvation on moulting, breeding, locomotion, and enemies. In his paper there is no mention of temperature, and the age of the animals was judged by their size. In 1915 Phipps made an experimental study of the behavior of amphipods with respect to light intensity, direction of rays and metabolism. *Hyalella knickerbockeri* was one of the animals included in his study. Gaylor (1922) reported on the life history and productivity of this species, and recently, the effects of population density upon its growth, reproduction and survival were studied by Wilder (1940).

In the above-mentioned investigations of *Hyalella azteca* no account exists of the morphological changes that occur in its postembryonic development, a necessary preliminary to experimental studies for which this animal seems suitable. In fact, few amphipods have been so studied. The work of Sexton (1924) on *Gammarus*

[1] A contribution from the Department of Biology, the Catholic University of America, Washington, D. C. This paper, prepared under the direction of Dr. Edward G. Reinhard, is based on the author's dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The writer wishes to express her gratitude to Dr. Reinhard who suggested the problem and gave constant assistance and encouragement throughout the work. To Dr. T. von Brand and Dr. W. G. Lynn thanks are due for helpful suggestions. The assistance of Mr. Clarence R. Shoemaker, Associate Curator of Marine Invertebrates, U. S. National Museum, who graciously placed at the author's disposal his fund of information on amphipods, is likewise gratefully acknowledged.

chevreuxi is undoubtedly the most outstanding contribution to our knowledge of the postembryonic development of amphipods. Sexton raised the amphipods individually from birth and studied their complete series of moults. When her work was begun in 1909 nothing was known as to the length of life of an amphipod, its moulting periods, incubatory period of the eggs, time required to reach maturity, etc. In addition to answering these questions for several species, she found the number of growth stages from hatching to sexual maturity to be different not only in the different genera, but different even in the various species of any one genus. *Gammarus locusta* lays eggs after the twelfth moult, *G. chevreuxi* after the seventh, and *G. pulex* after the tenth moult.

With the hope of adding to the knowledge of amphipod development, and keeping in mind broader biological problems, the question of growth and differentiation of *Hyalella azteca* was investigated according to the following plan:

1. Growth, from hatching to sexual maturity, considered with respect to the number of moults and the morphological differences between the instars.
2. Correlation between the differentiation of external secondary sex characters and the development of the gonads.

Materials and Methods

The Hyalellas were obtained from Mr. Eugene W. Surber, in charge of the Fish Hatchery of the U. S. Fish and Wildlife Service at Kearneysville, West Virginia. They are descendants of a stock originally collected by Mr. Surber in a Mississippi River slough near St. Charles, Missouri.

The study of growth, from hatching to sexual maturity, was investigated according to the following procedure: Young Hyalellas were taken on the day they emerged from the brood pouch of the mother and placed in individual culture dishes. For the newly-hatched animals ordinary Syracuse dishes were used. Bits of freshwater plants, such as Elodea and Vallisneria were added to the tap water and sand in the culture dishes. When the animals grew larger and there was danger of their crawling over the edge, larger dishes were substituted. The young animals were kept in a dark room where a constant temperature of 16 to 18° C. was maintained. Cultures were examined daily, and the moults preserved separately in vials of 5 per cent formalin until complete series from birth to maturity had been collected. Niagara Sky Blue, an aniline dye excellent for chitin, was used in staining the moults, and permanent mounts were made in Diaphane.

As soon as the first formation of eggs was observed in the young developing female, a male from a stock culture of mating males was added to her culture dish. Both the date of pairing and date of egg laying were recorded. The number of moults that preceded each of these events was likewise noted. Further observation was made in regard to the development of these eggs and the number of young produced in first broods.

When males could be identified by their large gnathopods, females (from the stock culture) with ripened eggs in their ovaries were added. Females were used that had just released young from their brood pouch and had new eggs ready to be fertilized. After pairing had taken place and eggs passed into the brood pouch, the eggs were observed for development, as this was the only means of determining whether or not the males were mature.

A comparison of the moults was made to study the earliest appearance and subsequent differentiation of secondary sexual characters. Brood plate development was considered in relation to growth of the ovary. Changes in the size and shape of plates as well as the time of the appearance of hairs were recorded. In the male, gnathopod differentiation was studied in its relation to the mating period. In addition, moults were studied for antennal growth and development of dorsal teeth.

General Observations

A. Description of Hyalella azteca (Saussure)[2]

The body of *Hyalella azteca* is elongated and laterally compressed, with the first thoracic segment fused to the head. The length of the animal is 4 to 6 mm. The thorax is composed of seven and the abdomen of six segments; the telson is small and entire. The eyes are sessile, compound, and round or nearly so. Antenna I is shorter than antenna II and is without an accessory flagellum; these features being characteristic of the family Talitridae. The peduncle of antenna I consists of three joints; the first and second joints are about equal in length and slightly longer than the third; the flagellum is composed of seven to nine joints, and is about twice as long as the peduncle. The peduncle of antenna II consists of five joints, on the second of which is located the antennal gland. The two distal joints of the peduncle are elongated and nearly equal. The number of joints in the flagellum of antenna II varies from eight to fifteen. Maxillae and maxillipeds will not be described as the study of their development does not enter into this investigation.

All thoracic legs, except the first and last pairs, bear gills on the inner side of the first joint. It is of interest to note that *Hyalella azteca* has two kinds of gills, sternal and coxal. Small lateral sternal gills are located on the thoracic segments III to VII inclusive, and the coxal gills project from the inner surface of the first joint of the thoracic legs II to VI inclusive. The first two pairs of thoracic legs differ from the others and are called gnathopods; the remaining five pairs are more or less similar in structure and are termed peraeopods.

Extending from the first three abdominal segments are three pairs of pleopods, each pleopod consisting of a long basal joint and two multiarticulated, setose rami. Pleopods are employed not only in swimming, but to direct water toward the gills. As the pleopods are constantly in motion even when the animal is resting, the gills and the developing embryos in the brood pouch are always aerated. A dorsal tooth projects from the posterior edge of each of the first two abdominal segments. The three posterior abdominal segments each bear a pair of uropods. These are directed backward and fitted for springing. The first and second pairs of uropods are biramous, and the third pair is uniramous.

1. *Males*

Males are distinguished from females by their larger size and by their second gnathopods. The propodus of the male's second gnathopod is very large, and the

[2] Smith gives a good picture of *Hyalella azteca* (=*Hyalella dentata*) in *Rep. U. S. Fish Com.*, 1872–73 (1874), p. 645, pl. 2, fig. 8.

palm is subchelate, in contrast to the female's which retains its juvenile small and chelate form. The testes, located beneath the heart, are much elongated and taper at each end. The male ducts open by papillae on the ventral side of the last thoracic segment.

2. Females

In females ova can be distinctly seen and approximately counted as they lie in the ovaries which are ventral to the heart. The paired tube-like ovaries extend from the second thoracic segment to about the middle of the seventh segment. When filled with eggs, they appear to terminate abruptly at each end. The narrow oviducts open at the base of the fifth coxal plates so that when the eggs are released they are caught in a brood pouch formed by the lamellae. Only during the passage of eggs can the oviducts be seen. In passing down, the eggs lose their ovoid shape and resemble a worm pressing its way forward. Immediately upon reaching the brood pouch the eggs assume their former shape.

The mature female can also be distinguished by lamellae, which occur on the inner surface of the coxal joints of thoracic legs II to V inclusive. The edges of the lamellae, except the posterior edges of the fourth pair, are bordered with hairs that are hook-shaped at their distal end. Hairs from adjacent lamellae entangle about each other and help to hold the eggs in the pouch.

B. Observations on mating

One of the characteristic features of the Amphipoda is their habit of pairing. Smallwood (1905) observed that even the sand fleas, *Orchestia palustris,* retain the habit of pairing, a very awkward process on land. Gaylor (1922) observed that Hyalellas pair for as many as seven days before copulation. In his discussion of sex recognition, Holmes (1903) states that pairing of the animals is determined by the reaction of the animals when they collide with each other.

Pairing is followed by the shedding of the female's moult, a necessary preliminary to copulation. Normally, the oviducts are closed externally, and temporary openings are only present after the moult when the cuticle is soft. The male then deposits sperm in the brood pouch around the orifices of the oviducts, and the eggs are fertilized as they enter the pouch. It may be mentioned here that the female Hyalella is able to release eggs into the brood pouch without the influence of the male, contrary to the statement of Embody. However, as such eggs are unfertilized, they apparently disintegrate and are eventually lost.

After copulation the animals cease swimming together. When a female bearing eggs in her brood pouch is found paired it is evident that the embryos in the pouch are well developed and will hatch in a few days, and the male is to become father of the next brood. Usually the ovaries are by this time filled with ripening eggs.

At the temperature at which Hyalellas were kept, about 21 days were required for development of the eggs in the brood pouch, i.e., from fertilization to hatching. After hatching, the young remain in the brood pouch for one to 3 days. One can observe the movement of their pleopods within the mother's pouch.

When ready to emerge, the young crawl to the front of the pouch which is open. After reaching the edge, they are abruptly thrown off as the mother makes a sharp

turn in her swimming movements. If the mother is again paired, the young do not remain in the brood pouch very long; the jerky movements of the male cause them to make an early exit. Some young were seen making their exit through the floor of the brood pouch by pushing the lamellae aside and tearing the hairs.

Instars Described in Terms of Moults

A. Four stages of development

Newly-hatched Hyalellas possess all the appendages of the adult, except the secondary sexual characters. Naturally, many modifications occur during the growth process. Unlike the amphipod, *Pontoporeia affinis,* in which the young possess male characters (Segerstrale, 1937), Hyalella young may be said to resemble the adult female, for the reason that the young have slender gnathopods like the female. Later, the males are distinguished by the increasing size of the propodus of the second pair of gnathopods.

In describing the growth stages of Hyalella, the instars may be conveniently divided into four characteristic periods: the Juvenile, the Adolescent, the Nuptial, and the Adult.

Table I

First occurrence of pairing determined for 20 males and 14 females in relation to instar age

Instar	5	6	7	8	9	10	11
Males.....	0	1	2	12	5	0	0
Females.....	0	1	0	13	0	0	0

The *Juvenile Period* includes the first five instars. First instar is that period extending from the time the animal emerges from the brood pouch to the time it sheds its first moult; assuming as seems likely, that no moult takes place while the young are still in the pouch. During the first five instars the sexes cannot be distinguished. The head of the newly-hatched animal is large in proportion to the rest of its body. Growth is slow and gradual, and the first antenna adds a maximum of three joints. In size and shape the propodi of the first and second gnathopods are similar. Dorsal teeth make their appearance in the third moult. With the shedding of the fifth moult the animal ends its Juvenile Period.

The *Adolescent Period,* including instars 6 and 7, shows an increased rate of growth in the entire animal. The sexes can now be distinguished. The propodus of the male's second gnathopod is much enlarged and the palm is transverse. The animals that retain the slender propodus at this period turn out to be females (with the exception of some retarded individuals). The paired tube-like gonads become more prominent and surrounded by adipose tissue. In some of the males the digestive tube, which is beneath the gonads, curves noticeably downward in the thoracic region and is lowest in the fourth, fifth, and sixth thoracic segments. In the seventh instar some of the females show the first formation of a few small eggs in the region of the fourth and fifth thoracic segments. Small brood plates are present but hairs are lacking on their borders. Another joint is added to the first antenna, and the fourth joint has again elongated and is ready to constrict.

The *Nuptial Period* designates the eighth instar during which the animals usually pair for the first time (Table I). Males pair almost as soon as the moult is shed, while the females generally pair toward the end of the period when the eggs in their ovaries have fully matured.

The animals are very much larger and the propodus of the male's second gnathopod is greatly enlarged with a distinct subchelate palm. One or two joints are added to the first antenna. The female's lamellae are turned back at the edges and a few hairs are present in the eighth moult which normally marks the end of the Nuptial Period.

The *Adult Period* occurs after the eighth ecdysis. Eggs are released into the brood pouch formed of fully developed lamellae bordered with hooked hairs. The eggs are fertilized by the sperm previously deposited around the orifices of the oviducts. Moult IX has an additional first antennal joint. When the ninth moult has been shed the animals have not attained full size. Growth continues beyond the tenth instar, but detailed observations were not carried on beyond this point.

TABLE II

Sample record showing duration of instars in days, Brood C, 12 individuals, emerged 12/8/42

Animal Instar.....	1	2	3	4	5	6	7	8	9	10
♀C1.....	8	6	13	12	11	10	16	(died)		
♂C2.....	8	17	5	10	15	11*	17	19		
♀C3.....	8	5	9	9	10	9	7	10*		
♀C4.....	9	26	12	10	8	12*	22			
♂C5.....	9	14	7	10	7	9	20	16*		
♂C6.....	9	13	11	8	9	9	10	12*	30	
♀C7.....	9	14	9	9	9	9	9	12*	25	
♂C8.....	10	14	9	8	9	10	12*			
♀C9.....	10	16	15	4	8	9	11	16*		
♂C10.....	11	15	10	6	9	10	9	24*	23	
♂C11.....	11	15	10	8	10	8	10	10	18*	
♂C12.....	12	12	8	10	9	9	12	17*		

The asterisk indicates the instar during which the animals paired for the first time.

Table II shows the duration of instars in days of the animals in Brood C.

B. General growth rate

At first growth is slow, moults I and II appearing to be the same size. The duration of the second instar is quite long; this is evidence of slow growth. In Figure 1 the time in days between moults is graphically represented for seven broods. Instars 3, 4, and 5 are short and during this period growth is rapid. Following moult V, differentiation of secondary sexual characters sets in. As the animals age they shed their moults less frequently. It is a known fact that young animals moult more frequently than do adults. Adult females moult more often than do adult males.

Body size in terms of moult length was determined for three representative males and two females, the measurements of the moults being made from behind

the head to the tip of the telson. The averages are seen in Figure 2. This graph shows that moults II, VI, and VII are larger in the females studied than in the males. However, general observations indicate that after attaining maturity the male grows at a faster rate than the female and as adult is characteristically larger

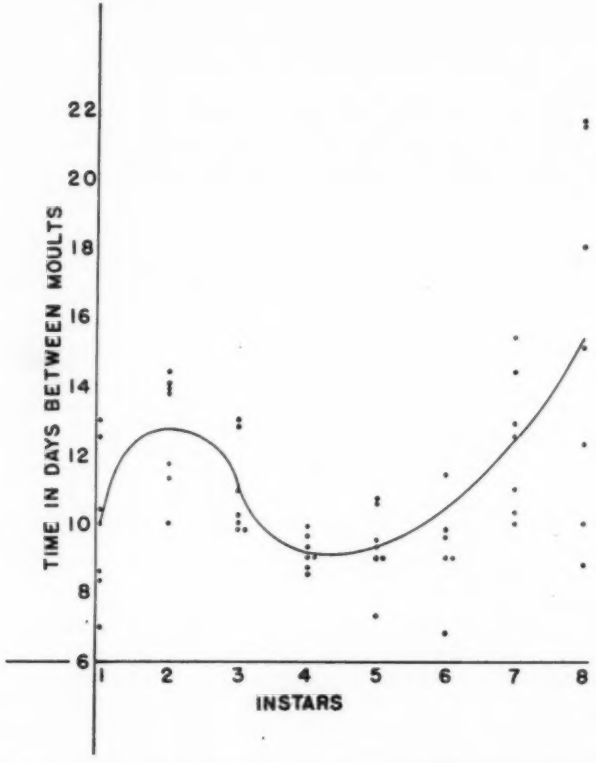

FIGURE 1. Time in days between moults measured for seven broods.

than the adult female. This does not appear on the graph since detailed studies in this case did not go beyond the eighth instar.

C. Growth of the antennae

Newly-hatched Hyalellas have six joints in the first antenna. The three proximal joints constitute the peduncle, and the distal three the flagellum. During the process of growth the number of peduncular joints remains constant while the flagellum develops four to six new joints. The actively growing region ("growth center") is the first proximal joint of the flagellum. Growth can readily be studied through the series of moults from any one animal. A number of series were studied

to compile the following table which refers to number of joints in the entire first antenna:

Moult	I.	6 joints; No. 4 is long.
Moult	II.	6 joints; No. 4 is long. Or 7 joints with No. 4 short and lacking setae. The constriction has just occurred, and setae which are normally found on the distal edge of each joint, have not yet developed.
Moult	III.	7 joints; No. 4 is short and lacking setae.
Moult	IV.	7 joints; No. 4 is long, and has setae. Or 8 joints with No. 4 short and lacking setae.
Moult	V.	8 joints; No. 4 is long, and has setae. Or 9 joints with No. 4 short and lacking setae.
Moult	VI.	9 joints; No. 4 is long, and has setae. Or 10 joints with No. 4 short and lacking setae.
Moult	VII.	10 joints; No. 4 is long, and has setae.
Moult	VIII.	11 joints; No. 4 is long, and has setae. Or 12 joints with No. 4 short and having setae.
Moult	IX.	11 joints; No. 4 is long, and has setae. Or 12 joints with No. 4 long and having setae.

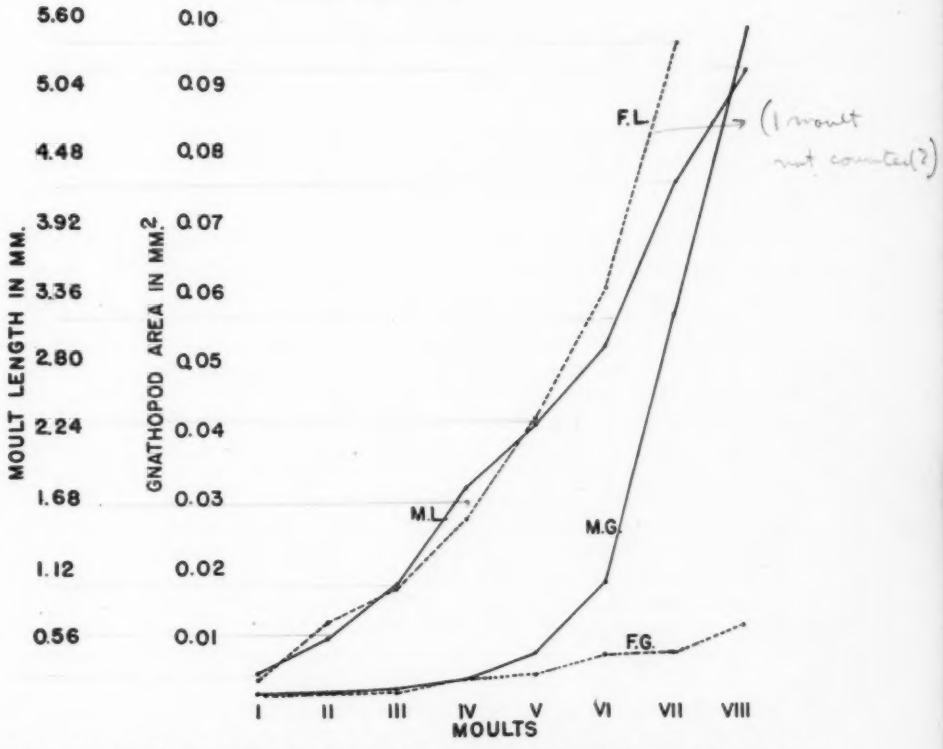

FIGURE 2. Growth in length of body of *Hyalella azteca* plotted against growth in area of gnathopods (propodus and dactylus). M.L.—length of male moult. F.L.—length of female moult. M.G.—area of male gnathopod. F.G.—area of female gnathopod.

In the early stages of development growth is relatively slow. With the shedding of two moults only one joint is added. From Moult V onward, joints are added at the rate of one per moult. Plate I is a representative series of drawings from moults shed by the animal D8. The outside view of the right first antenna is drawn to scale.

It cannot be said that any particular moult always has a certain number of joints, because the growth rate varies in different animals. Sometimes in the same moult one of the first antennae may have one joint more than the other first antenna (Pl. III). In such a moult it will be noted that in the one case the first proximal flagellar joint has lengthened and constricted, and in the other case, the corresponding joint has lengthened; due to a slower rate of growth, it has not yet constricted. In general, however, the number of flagellar joints is helpful in ascertaining the age and instar of the animal. An animal with a six-jointed first antenna cannot be beyond its third instar; if the animal has nine or ten joints, it is not yet mature, but is passing through one of the adolescent instars.

Second antennae—method of multiplication

As in the case of the first antenna, the "growth center" or region of most active growth of the second antenna is located in the first proximal joint of the flagellum. There are five joints in the peduncle and therefore the first joint of the flagellum is No. 6.

Moult I has a total of nine joints and this number does not increase in the second and third moults, although joint No. 6 elongates preliminary to constriction. Moult IV has an additional joint. In moults V to IX the number of joints steadily increases from 11 to 15.

In *Hyalella azteca* only one region of growth could be detected in the antennae, but in some amphipods more than one growing region is present. Segerstrale (1937) found this condition in the male *Pontoporeia affinis*. In the beginning, only the proximal joint of the flagellum divided, but in the third last stage, four of the more distal joints divided, and in this same stage the proximal joint divided three times. In the stage before the last, 15 proximal flagellar joints divided, so that the last stage of the male has a flagellum of 41 joints. The female of this species is distinguished by a shorter flagellum. Only the proximal joint divides. In the stage before the last there are ten joints, and the last stage shows no increase.

In *Gammarus chevreuxi* Sexton (1924) found that the proximal joint of the first antenna divides, usually into two portions, and the distal one which is the new joint she terms the "undivided primary joint." At the next moult the undivided primary joint elongates and divides into two portions, the distal part or true primary joint and the proximal portion which may be called the secondary joint. The process is repeated in each stage, the last formed primary joint subdividing and giving rise to a secondary joint; the formative zone dividing and giving off one or

PLATE I

Development of first antennae is shown above. The right first antenna is drawn to scale in outside view from the first eight moults shed by one animal. Arrows indicate growth pattern, i.e. the manner in which successive new joints are added by division of the fourth joint. Development of propodus and dactylus of first gnathopods of a female is shown below. Drawings made to scale from the first eight moults shed by a female.

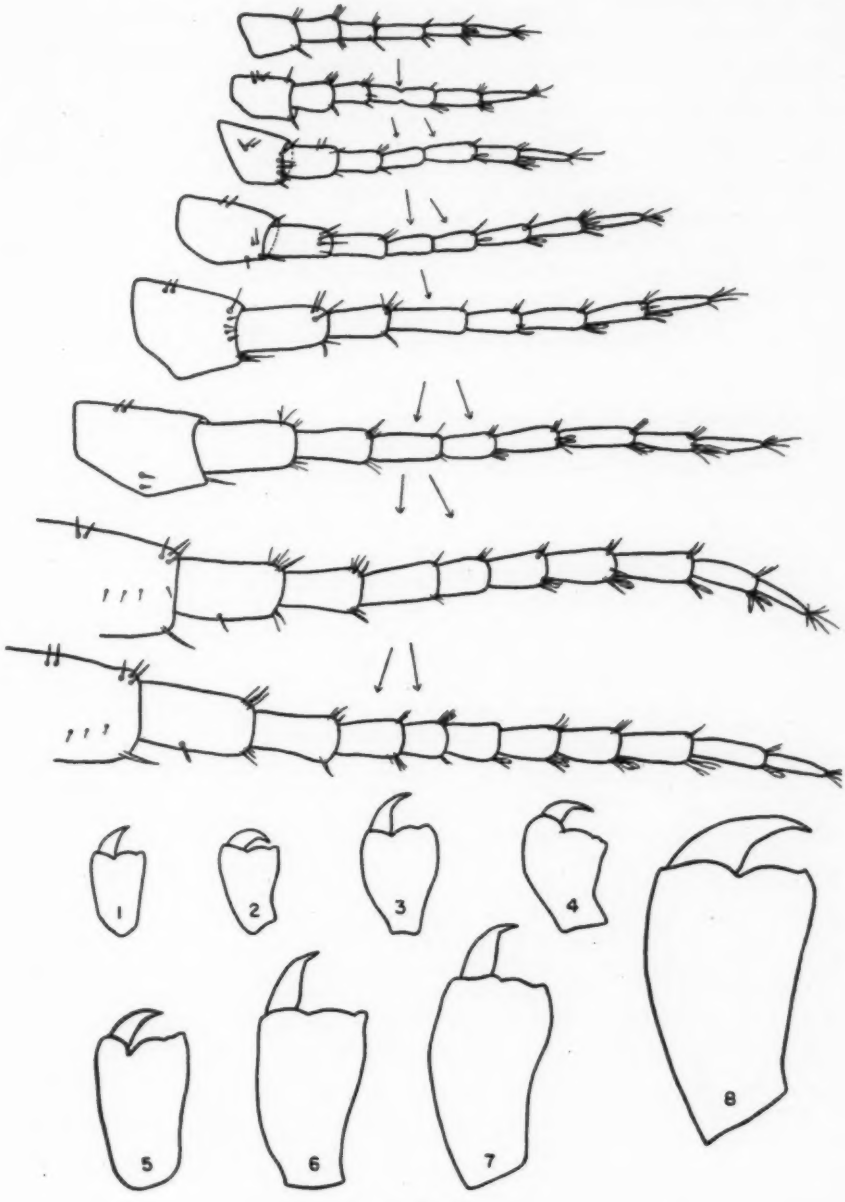

PLATE I

more undivided primary joints. Sexton also mentioned that sometimes more than one primary joint may be formed in the older animals. In *G. chevreuxi* the second antenna has a shorter flagellum than that of antenna I, and is slower also in the rate of growth.

Growth of the first antenna of *G. chevreuxi* is more rapid than that of *H. azteca*, for in moult I of the former there are seven joints in the antenna and in moult VIII of the same animal there are 23 joints. In moult I of *H. azteca* there are six joints in the first antenna and in moult VIII there is a maximum of 12 joints.

While the second antenna of *G. chevreuxi* is slower in its rate of growth than the first antenna, the rate of growth in the second antenna of *H. azteca* is about equal to that of its first antenna for six additional joints are usually present at the eighth moult.

Development of Secondary Sexual Characters

A. Gnathopods

In the early instars the propodus of the first and second gnathopods in both sexes is approximately the same size, but distinctly different in form. The palm of the propodus of first gnathopods is transverse and concave at the middle. Although this propodus increases in size with the age of the animal, it does not alter in form in either sex. A series of propodi of first gnathopods of a female, C13, is shown in Plate I. These were drawn to scale from the mounted moults of the animal. The first gnathopods of the male have similar propodi.

In contrast, the second gnathopods become sharply differentiated as the sexes mature. In both males and females the palm of the propodus of the second gnathopods is chelate during the juvenile period, and in the female the juvenile form is retained throughout life, even after the propodus has become long and slender. The complete series of propodi of second gnathopods of C13, also drawn to scale, are shown in Plate II and indicated by the letter "F."

After the fifth moult, the propodus of the male's second gnathopod gradually changes both in size and form. The palm loses its chelate form and gradually becomes transverse. From about the seventh moult on, the palm assumes a pronounced subchelate form. The dactylus of the male curves more strongly to fit the changing form of the palm. In Plate II the series of propodi indicated by the letter "M" are from male second gnathopods. All are from the male C12, except No. 3, No. 5, and No. 6 which are from the male B1. Moults III and V of C12 were badly torn, and moult VI was unusually small and not representative of a typical sixth male moult, so in the drawing the propodus from B1 was substituted.

Hundreds of male moults had been collected in an attempt to secure a complete series from one animal. Although Hyalellas do not eat their moults, they sometimes tear them badly in the process of moulting. In many cases, moults that had been preserved were found to lack both propodi from the second gnathopods, the very feature that was most essential to this study. In moulting, the male has to pull his immense propodus through the narrow carpus and in the struggle the

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Plate II

Development of propodus and dactylus of the second gnathopods. Drawings made to scale from the first eight moults of a female (F) and male (M).

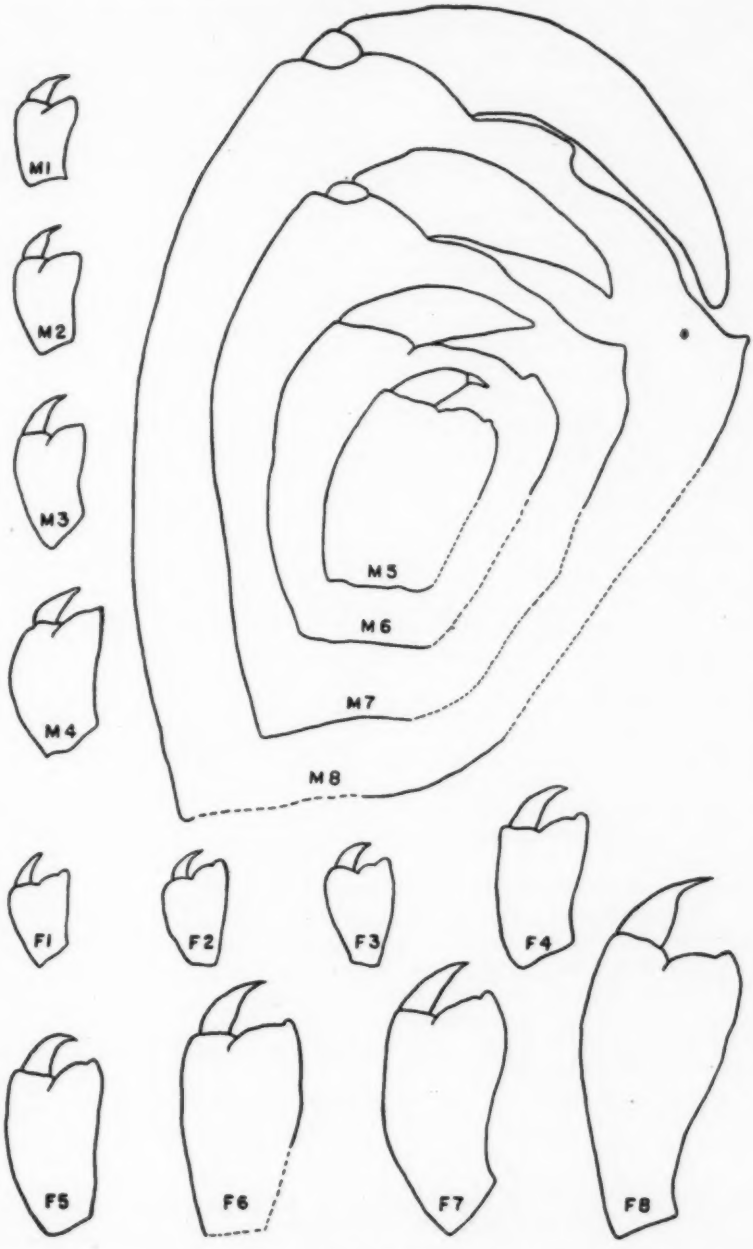

PLATE II

propodi are often torn off. In nearly every propodus that is retained the sternal border is torn.

To obtain some idea of the increase in area of the propodus of both males and females, series of drawings to scale were made, and the actual area of the gnathopod was calculated from these drawings. A complete series of propodi from one female was used, but as there was no complete series of male propodi, an average obtained from the propodi of three males was used in determining area. The data are presented in Figure 2.

The propodus of the male in moult V is 4.7 times the area of the same propodus in moult I; in moult VI, it is 11.8 times, in moult VII, 37 times, and in moult VIII, 64 times. The female propodus shows a gradual increase; in moult VIII it is 8 times the area of the same propodus in moult I (Pl. II).

In *Hyalella azteca* the gnathopods differentiate as external secondary characters before the animals are sexually mature. When males could be identified by their gnathopods as early as the sixth instar, mature females with ripe eggs in their ovaries were added. At this stage the males were not mature, because they did not pair. When those same females were removed and placed with mature males from the stock culture, pairing immediately took place and was followed by copulation.

After the young males shed their next moult and were then in their seventh instar, mature females were again added. In a very small percentage of cases pairing took place. In general, pairing took place after males had shed seven moults. The fact that young hatched from those eggs shows that they were fertilized. Although males may be regarded as sexually mature in their eighth instar, in five cases males first paired during the ninth instar (Table I). In these instances the females that were added during instar 8 may not have been in the right physiological condition for mating.

If we consider pairing as an indication of maturity, both sexes are normally mature in the eighth instar. On the other hand, if the instar during which ova deposition occurs is taken as the first sign of sexual maturity then the female is mature in the ninth instar, because her eighth moult must be shed before eggs are released. The male, in the latter case, may be said to mature one instar before the female, because he is capable of fertilizing eggs immediately after pairing and before his eighth moult is shed.

The first gnathopods of the male Hyalella are employed in carrying the female and are structurally adapted for this purpose. The fact that the second gnathopods enlarge when the male approaches maturity seems to indicate that they are necessary for sex recognition. Males need them to actively resist each other, and females in lacking them are not able to resist being carried, as described by Holmes (1903). Sexton observed that *G. chevreuxi* male carries the female with his second gnathopods, and Heinze (1932) stated that *G. pulex* male uses his first gnathopods for this purpose.

After reaching maturity, the male continues to grow, and the second gnathopods increase in size proportionately, until the maximum adult form is reached.

B. Lamellae

Lamellae, characteristic of adult females, are entirely lacking in the young animals. The sixth moult of females has small, incompletely developed lamellae; the

seventh has them slightly enlarged. In moult VIII the edges of the lamellae are turned backward and bordered with a few scattered hairs. Moult IX contains the fully developed lamellae bordered with hairs; this is the first adult moult of the female.

The hairs differ from the long, straight hairs found in Gammarus and certain other amphipods. In Hyalella, as in other members of the family Talitridae, the basal portion of the hair is slightly enlarged, and the distal end is swollen and curved around in the shape of a hook. The hooked ends join with hairs from opposite or adjacent lamellae to help hold the developing eggs in the pouch (Pl. III). When eggs are first deposited in the pouch, opposite lamellae overlap each other. In later stages, when the embryos are large, the lamellae are fully stretched out and only the hairs cling together. Perhaps the increased weight or the movement of the young push the lamellae out to their full extent. Small, young females generally have fewer eggs than older females which are larger, and the size of the lamellae is in proportion to the size of the animal. Four to 13 young were produced in first broods.

The fourth pair of lamellae is smaller than the three anterior pairs and the posterior margin, which is slightly chitinized, is curved upward. Hairs are lacking along that same border. Due to the upward curvature of the lamellae, it may be that hairs are not necessary. A few spines are located on the external side of the curved portion.

Development of hairs is in some way correlated with maturing of the eggs, for they appear and function for the first time when the first eggs are released.

The development of lamellae in Hyalella is similar to that of other amphipods. Sexton reported that in *G. pulex* where the cuticle is firmer and more substantial than in *G. locusta* or *G. chevreuxi,* she has been able to trace brood plates in four growth stages preceding maturity. First they appear as minute, rounded leaf-like plates with margins entire; in the next moult they have lengthened and increased to three times the size; rudimentary hairs make their appearance on the next moult, and in moult X (the moult after which eggs are laid) brood plates are very large, chitin is hard, and rudimentary hairs more numerous. In the next stage when sexual maturity is reached, the brood plates are fully developed with long fringing hairs. In *G. locusta* and *G. chevreuxi* Sexton traced brood plate development in three stages before sexual maturity.

Heinze, too, from his study of *Carinogammarus Roeselii* Gerv. concluded that the first anlagen of oostegites appears several moults before the female becomes mature. In fact, he found the first pair of lamellae on the third segment when the animal was 4.5–5 mm. Heinze does not mention the instar of the animals, but gives their length in millimeters. When the female is 5–5.5 mm. long the four pairs of brood plates are present, the fourth pair on segment 6 being the narrowest. The closer the female approaches maturity, the larger the brood plates become. In stages 7.5 mm., 8 mm., 8.5 mm. the hairs are still lacking, and in the next stage, bordering of the brood plates with hairs precedes copulation. In *G. pulex* Heinze found similar conditions, the first pair of brood plates appearing when the animal is 4.5 mm. But corresponding to the larger size of the female, the oostegites in their development were more advanced.

Segerstrale reported that the first anlagen of brood plates in *Pontoporeia affinis*

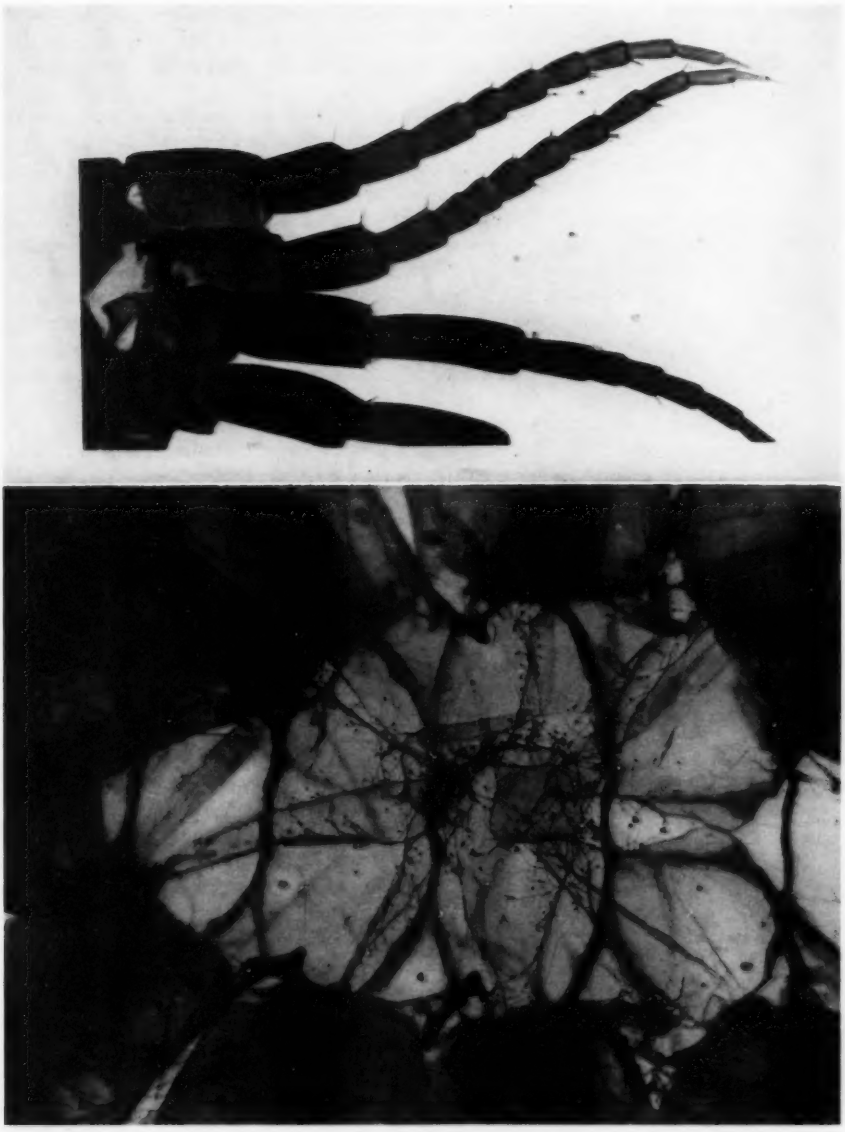

PLATE III

FIGURE 1. A case of unequal growth of the first antennae. A constriction has appeared in the first proximal flagellar joint of the right antenna, while the same joint in the left antenna has elongated but not yet constricted. × 68.

FIGURE 2. Photomicrograph of female moult to show brood plates with interlocking hairs as seen from within. × 68.

occurred in females that were 5.3–5.5 mm. in body length. They appear as small rounded structures which expand by degrees so that in the stage before the last they show approximately the final form. The enlargement of the area occurs in the greatest degree only during the last moult.

Retarded Individuals

During the course of this investigation, it soon became apparent that all the animals did not mature at the same time. This was first noticed in the animals that were raised individually. In the eighth instar the animals generally paired, showing that they were sexually mature. About one or two in some broods failed to pair at this time, due to their immature condition. For the present, since their precise nature is unknown, these slow-developing animals will be termed "retarded individuals." At a stage when their siblings could readily be distinguished by their gnathopods, and also by eggs in the case of the females, these retarded individuals, while possessing the slender gnathopods of the female, lacked visible eggs. Retarded individuals are smaller than the rest of the animals in the same brood and grow at a slow, steady rate. At a much later stage some of them gradually developed male gnathopods and eventually paired. Two others after the tenth moult and therefore during the eleventh instar, developed eggs and later paired. One of them lost its first brood of eggs, but its second brood, which had been fertilized by a retarded, but now mature male, developed into young in the normal length of time. However, none of the young reached maturity.

Two retarded individuals, aged 67 days (the rest of their brood had matured at 40 days), when sectioned and stained, showed a few very small eggs in their gonads. Another, having female gnathopods but lacking eggs, was dissected. It was found to have large, normal brood plates that lacked hairs.

It is possible that the retarded individuals are similar to the intersexes described so completely by Sexton. A thorough study of them, outside the scope of the present investigation, would have to be made to determine their exact status. It would be interesting to learn whether a deficiency, hormonal or nutritional, prevents maturation of the gonads at the normal time, whether their genetic constitution warrants a later maturation, or whether retardation may be caused by a state of depression induced by unfavorable external factors. A study of this special problem is now under way.

Summary

Hyalella azteca grows well under laboratory conditions. Like other amphipods, the frequency of its moulting depends upon several factors, chief of which is temperature. At low temperatures ecdysis is delayed. Frequency of moulting in developing animals is no indication of sex, as it occurs at varying time intervals in both sexes. The young moult more often than do the adults. In reaching the time for first pairing, the number of moults is more important than the age, in days, of the animal. First pairing generally occurs in the eighth instar.

In general, growth at first is slow. After the secondary sex characters first become apparent (Instar 6) growth becomes more rapid.

Centers of more active growth are present in certain regions, such as the second gnathopod of the male, and the proximal joint of the flagellum in both pairs of

antennae. In the female the fifth thoracic segment appears to be a "growth center," for eggs begin forming in this region. No doubt, there are regions of active growth throughout the body, but the above are the most prominent.

Animals during any instar are not the same size. Males particularly vary in size, although they may mature after the same moult. Retarded individuals, animals that pair much later than the normals, are fairly common. It is possible that some of them never become sexually mature.

The external secondary sex characters, lamellae and gnathopods, make their appearance several moults before the animals pair. Lamellae grow gradually, and reach their full size and become bordered with hooked hairs when the first eggs are released.

First gnathopods in both sexes are similar in size and form. Second gnathopods of the female retain their juvenile form, although they increase in length. Second gnathopods of the male develop a very large propodus with a strong dactylus and the palm of the propodus changes from chelate to transverse and then finally to subchelate.

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CHANGES IN WEIGHT AND WATER CONTENT DURING THE LIFE CYCLE OF THE WOOD-EATING BEETLE, PASSALUS CORNUTUS FABRICIUS

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Introduction

That relative humidity and water relations in general are important in the lives of insects has been well established. The literature on the subject is voluminous. Excellent reviews by Buxton (1932), Mellanby (1935), and Johnson (1942) discuss insect water relations from various points of view, such as saturation deficiency and longevity, evaporation of water, the humidity of the environment, etc. Others (e.g. Johnson, 1937; Slifer, 1938) have studied the mechanism and rate of water absorption in the insect egg. Ludwig (1931), in reviewing the literature on the subject of changes in weight and water content during metamorphosis of insects, calls attention to the fact that in different species entirely opposite phenomena may take place at corresponding stages in development. To give one example: Bodine and Orr (1925) observed that in Drosophila a marked *increase* in weight occurred on emergence of the adult, while Ludwig (1931) found that during the metamorphosis of the Japanese beetle from larva to adult there was a *loss* of weight amounting to half the maximum larval weight. In both cases the changes in weight were attributed mainly to changes in water content. Thus it would seem that no hard and fast rule can be laid down that will apply to all insects; the story must be worked out independently for different species living under different conditions.

In this paper an attempt is made to establish the normal weight and water content of a beetle, *Passalus cornutus*, at all stages in its life history from egg to adult under stable conditions. A study of the ranges of tolerance to humidity and temperature are left for a later paper. Among the beetles there are few, if any, records of the changes in water content of a species throughout its whole life cycle. Ludwig's (1931) studies of the weight changes and water relations of the Japanese beetle larva, pupa, and adult offer the most satisfactory comparison to the conditions found in Passalus, although the habitat and duration of the life cycle of the Japanese beetle and of Passalus differ markedly.

Several factors make Passalus a convenient insect for study: large size, abundance, ease in obtaining, large size of eggs, and short life history. Passalus is a wood-eating beetle which spends practically all of its life in decaying hardwood logs. The adult beetles make extensive tunnels in the logs; and here the eggs are laid, and larval, pupal, and adult life is lived. The eggs are often deposited in an offshoot of a tunnel, out of the way of the main line of traffic. Although not all eggs of one female are laid at the same time, all her eggs are laid in the same place in the runway. Always they are on, and surrounded by, an abundance of moist,

finely chewed frass prepared by the adults. Passalus is a social beetle. The adults protect the developmental stages, prepare frass for the larvae, and build cocoons for the pupae. The developmental cycle is completed in a relatively short time for so large a beetle. In the vicinity of Durham, N. C., eggs are laid in late May or June and new adults appear in August.

Only hardwood logs that have a high percentage of moisture are inhabited. In fact, the whole life cycle must of necessity be completed in a moist environment. Data at hand indicate that eggs will not hatch nor larvae develop except when in contact with moisture. Even the adults cannot live at low humidities, and the adults are more hardy in this respect than are the eggs, larvae, and pupae.

Materials and Methods

Passalus eggs of known age were obtained in the laboratory in the following manner. Laying females were brought in from the woods and placed in finger bowls with some of the frass from the logs. One female was placed in each finger bowl. The dishes were covered and kept in a dark cabinet. Each morning the frass was examined for eggs. When first laid the eggs are bright red in color and are not difficult to distinguish from the frass. As Passalus seldom lays more than two eggs in a 24-hour period, and as all females brought in do not lay in the laboratory, it takes a great many beetles to obtain a sufficient supply of eggs for experimental purposes. The eggs were placed in petri dishes containing either moist filter paper or moist frass. Volumes of the eggs were determined by the formula $V = \frac{4}{3}\pi a^2 b$, where a equals the shorter semi-axis and b equals the longer semi-axis. The calculations are probably not absolute, for all eggs are not perfectly ovoid, but volumes computed by this formula are relative and give very satisfactory comparisons. Measurements of the eggs were made to the nearest tenth millimeter with a vernier caliper. In all, several thousand eggs have been used in various experiments over a period of several years.

All stages in development were kept in an insulated cabinet at 27° C. Larvae, prepupae, and pupae were kept in large covered dishes with an abundant supply of frass and moisture. Larvae of different ages were kept in separate dishes to prevent the larger larvae from injuring and eating the smaller. Adults were not kept with the developmental stages. All weighings were made on a chain-o-matic balance. To determine water content, from 15 to 25 eggs of each age group were dried in a vacuum oven at 98° C. until the weights became constant.

Observations

Changes in weight, volume, and water content of the eggs

Figure 1 shows graphically the close correlation between weight and volume of Passalus eggs at different age levels. The data for these curves were obtained from material collected during a single season and kept under similar conditions. About 350 eggs are represented. All were weighed and measured individually, some each day, others every other day. That the increase in weight is due to an increase in water content is clearly shown in the parallel curves of Figure 2. The eggs represented here are not the same as those used for plotting the curves of Figure 1 and were collected in a different season. In each age class 15 to 25 eggs were weighed

collectively, dried, and reweighed. The curves indicate that there is a slight decrease in dry weight as development proceeds.

Passalus eggs are large as insect eggs go, and are ovoid in shape. When first laid they measure approximately 3.20 mm. × 2.50 mm. and increase in size to about 3.80 mm. × 3.20 mm. at time of hatching. Newly laid eggs weigh between 10.0 and 12.0 mgs.; at time of hatching between 18.0 and 21.0 mgs. There is of course considerable individual variation in size and weight of the eggs. The amount of water in the eggs increases from 7.25 mgs. in newly laid eggs to over 15 mgs. in those ready to hatch. In per cent the water content increases from 68 to 83. Weight, volume and water content do not show marked changes for the first five

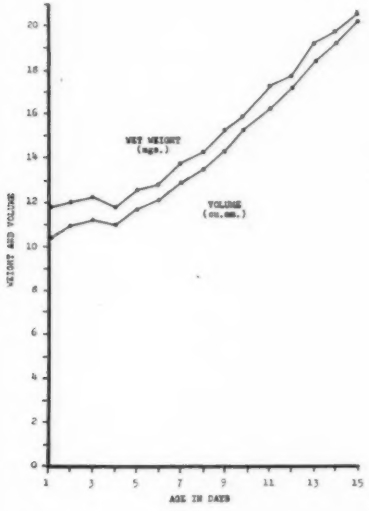

FIGURE 1. Changes in wet weight and volume during development of the eggs of *Passalus cornutus.*

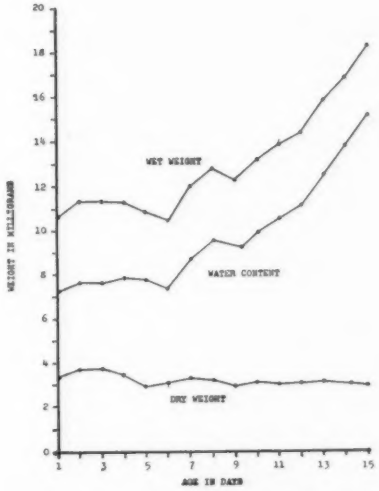

FIGURE 2. Changes in wet weight, dry weight, and water content during development of the eggs of *Passalus cornutus.*

days of development; but following this initial period of relative stability, weight, volume, and water content increase rapidly and continue to do so up to the time of hatching, which at 27° C. requires 15 days. Changes in color accompany the changes in weight, volume, and water content. Newly laid eggs are bright red. This gradually turns to a reddish-brown during the first week of development, then to a brownish-green, and within a few days of hatching to dark green. The significance of the color changes has not been determined.

Changes in weight and water content of larva and pupa

There are three larval instars. In the laboratory the first lasts about 16 days, the second about 13 days, and the third (including the prepupal stadium) about 28 days. Near the end of the third instar the larvae cease feeding and wander about

for a few days seeking a place to pupate. Entrance upon the prepupal stadium is gradual and is determined with difficulty. As soon, however, as the prepupae become sluggish and take on their characteristic white, fatty appearance, they can then be readily distinguished from the active, bluish larvae that are still feeding. The prepupal stage lasts 5 to 6 days.

On hatching from the egg the larvae vary in length between 8.5 mm. and 11.5 mm. and weigh between 18.0 mg. and 23.0 mg. Little or no food is taken for the first day or so, but after feeding is well under way—in 24 to 36 hours—the color changes from white to bluish and the increase in size and weight is rapid. There is great individual variation, but the weight of a larva may increase to as much as

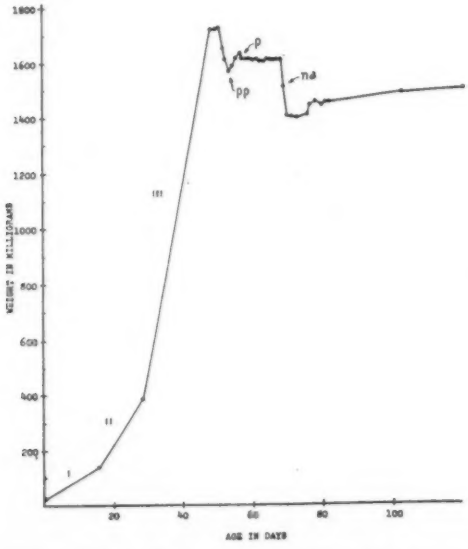

FIGURE 3. Changes in weight of *Passalus cornutus* from larva to adult. I, II, III, the three larval instars; pp, beginning of prepupal stage; p, beginning of pupal stage; na, newly transformed adult.

140 mg. by the end of the first instar, 400 mgs. by the end of the second instar, and 2000 mgs. by the end of the third instar. Thus the weight may increase a thousandfold in less than 2 months. The weight falls slightly after the late third instar larvae cease feeding and enter the prepupal stage; it rises slightly just before pupation begins; and again falls off, this time abruptly, on emergence of the adult. The weight of the adult is about five-sixths that of the greatest weight of the larva. Individual weight records were kept on many larvae raised in the laboratory and carried through to adulthood. A typical weight curve is given in Figure 3. Among the adults, as in all other stages, great individual variation in weight occurs. There is no sexual dimorphism, but the average female is larger and about 12 per cent heavier than the average male. The average weight of 27 old (not newly

emerged) males was found to be 1478 mgs.; and of 35 old females, 1686 mgs. The range in weight of males was from 977 mgs. to 1740 mgs.; of females, from 1390 mgs. to 1972 mgs.

Newly hatched larvae contain about 85 per cent water. As soon as the larvae begin feeding the water content rises to about 90 per cent. This percentage is maintained until the larva ceases feeding and enters the prepupal stage when it falls to 88 per cent. There is then a steady increase in percentage of water until a high of 93 per cent is reached in the late pupal stage. The changes in water content in the prepupa coincide with the changes in weight, but in the pupa the weight remains constant while the percentage of water increases. A loss in dry weight is apparent as the internal tissues undergo histolysis. On emergence of the adult the per cent of water does not fall abruptly to the low level of old black adults, but declines gradually. The total weight of the adult, however, remains essentially constant so that the gradual decrease in percentage of water is explained by a gradual

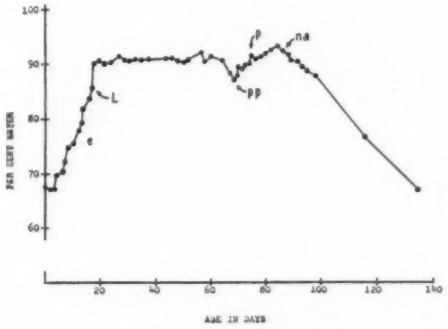

FIGURE 4. The water content, in per cent, throughout the life cycle of *Passalus cornutus*. e, egg; L, newly hatched larva; pp, beginning of prepupal stage; p, beginning of pupal stage; na, newly transformed adult.

increase in dry weight. The red color of newly emerged adults persists for several weeks and may last for several months, but as the red gradually gives way to brown and the brown to black, the percentage of water falls until it finally reaches the level of 67 per cent for old adults. Twelve adults a month after emergence were found to still contain as much as 76.5 per cent water. No appreciable difference in the water content of males and females was observed.

A curve of the changes in percentage of water content from egg to adult is shown in Figure 4.

DISCUSSION

That Passalus eggs should absorb water is not surprising for this phenomen has been observed in other insect eggs. Bodine (1929) was among the first to show definitely that the increase in weight of an insect egg is due to an actual increase in water content. Slifer (1938) has given a brief history of our knowledge of this subject and in her own experimental work has demonstrated the manner in which

water is absorbed by the grasshopper egg. She has shown that the ability to absorb water coincides with the formation of a special structure at the posterior end of the embryo. This she has designated as the hydropyle. In the eggs of *Melanoplus differentialis* there is no water absorption for the first 6 days of development (until the hydropyle has formed); then there follows a period of gradual increase in water content which lasts until several days before hatching, when the eggs become turgid and water absorption ceases. The shape of the weight curve presented by Johnson (1937) for Notostira indicates that the pattern of water absorption in the eggs of this insect is similar to that of the grasshopper: there is a lag of a few days followed by a rapid increase in weight (and water content); then a marked decline in the rate of increase occurs several days before hatching.

The curves of increase in wet weight and water content of Passalus eggs are similar to those of the grasshopper and Notostira except in one particular. In Passalus eggs water uptake begins about the fifth day, as in the grasshopper egg, but unlike either the grasshopper or Notostira the absorption of water continues up to time of hatching. The curve does not level off a few days before hatching time as in these other insects. If present at all, the "leveling-off period" must be very brief in Passalus eggs. Failure to demonstrate it cannot be explained by lack of data, for none of the many eggs, whose individual daily records of weight and volume were kept from time of laying to hatching, showed leveling-off in either weight or volume.

There is evidence to indicate that a hydropyle is formed in Passalus eggs. In fixed eggs a small ring is apparent at the posterior end of the egg about the fifth to sixth day of development, and its appearance can be correlated with the beginning of water uptake. Studies are in progress on the histological structure of the hydropyle of the Passalus egg.

As is to be expected the curve of egg volume parallels the curve of wet weight. The exact age of the egg cannot be determined by volume alone because of the large individual variations in the eggs. One can, however, estimate with a fair degree of accuracy whether an egg of unknown age is in its first, middle, or last third of development. Size and color together make fair criteria for roughly determining the ages of eggs found in logs.

The eggs practically double in wet weight, volume, and amount of water during their development. The contentions of Bodine (1929) and of Johnson (1937) that the gain in weight is due to water uptake are supported. Johnson (1937) has suggested that there is actually a decrease in dry weight of the eggs as development proceeds. It seems probable that this may be true, for in Passalus eggs there is a slight but noticeable downward trend in dry weight. However, at least in Passalus, a larger number of eggs should be used before drawing a definite conclusion on this point. The loss in dry weight is gradual and is probably caused by the transformation of the large amount of yolk into food materials and protoplasm.

In Passalus, while the curves of weight and water content of larvae, prepupae, and pupae follow the same trend as found by Ludwig (1931) for the Japanese beetle, some deviations worthy of mention occur. The time spent in larval stadia is much shorter in Passalus and growth is much greater and much faster. It is not uncommon for larvae to make a 10,000 per cent increase in weight in less than 2 months. Evidence indicates that under natural conditions in the logs in the forest,

where temperatures often rise above laboratory temperatures, growth is even faster than reported in this paper. The water content of the larva is definitely higher in Passalus. It averages about 90 per cent in contrast to 78 per cent in the Japanese beetle larva. In both larvae a drop in weight occurs in the prepupal stage. In Passalus, however, the fall in weight begins as soon as the larva stops feeding and before it definitely enters the prepupal stage and is followed by an increase in weight before the pupal stage is reached. The increase in per cent of water in the prepupa and the pupa is readily accounted for by the degenerative changes of the larval tissues which take place in these stages during metamorphosis. The pupae, especially, are extremely delicate and in their natural runways would not last long were it not for the heavy protecting cocoon built around each by adults. In the late pupa the water content reaches a high of 93 per cent. This is above that of the larva. In both the Japanese beetle and Passalus a sharp drop in weight is apparent on emergence of the adult. In Passalus, however, the decline in weight is not nearly so great as in the Japanese beetle and sometimes amounts to no more than one-eighth the maximal larval weight, although usually it is about one-sixth. This is in marked contrast to the Japanese beetle, whose loss in weight amounts to one-half the maximum larval weight. Evidently the decline in the per cent of water in newly emerged adults of Passalus is more gradual, and coincides with the gradual increase in dry weight. Sclerotization is slow. New adults are very soft and remain within the cocoon for several days before venturing forth to compete with other members of the colony. The slow increase in dry weight is correlated with the slow and gradual changes in color and in degree of sclerotization that the adults undergo. The change from pink to red to brown to black usually requires a month or more, but may require several months. Eventually the beetle becomes black. When it does so the water content has reached the level of 67 per cent common to the old adults and the integument has become thick and hard. Thus, in percentage, the water content of the adult Passalus eventually gets back to its starting point in the egg.

Summary

The changes in weight and water content from egg to adult have been shown for the wood-eating beetle, *Passalus cornutus*.

In the egg little change occurs for the first 5 days of development; then the weight of the egg, the volume of the egg, and the amount of water increase steadily until hatching. The increase in weight is due to water uptake. As weight, volume, and water content increase the color of the egg changes from bright red to dark green.

During larval life, which in the laboratory at 27° C. lasts approximately two months, the weight may increase more than 10,000 per cent. A slight fall in weight occurs when the larva ceases to feed and enters the prepupal stage. On emergence of the adult there is a sharp drop in weight amounting to about one-sixth the maximum larval weight.

The water content of a newly laid egg is about 68 per cent. This increases to 83 per cent at time of hatching. Newly hatched larvae contain about 85 per cent water, but after feeding begins this increases to about 90 per cent. A slight fall in percentage of water occurs when the larva ceases to feed, but increases again in the

prepupal and pupal stages until, in the late pupa, it reaches a high of 93 per cent. The fall in per cent water is gradual in newly transformed adults and several months may be required for it to reach the level of 67 per cent characteristic of old adults.

The gradual change in water content of the new adults is correlated with the slow increase in dry weight, slow sclerotization of the integument, and with changes in color from red to black.

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THE QUESTION OF THE SYNTHESIS OF THIAMIN BY THE CILIATE, GLAUCOMA PIRIFORMIS

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Even in the absence of conclusive evidence it has sometimes been assumed that thiamin is synthesized by the plant-like flagellates, either from relatively simple substances or from the thiazole and pyrimidine components of this vitamin. Specific evidence that *Chilomonas paramecium* synthesizes the diphosphopyridine nucleotide (Hutchens, Jandorf and Hastings, 1941) has furnished a real basis for the assumption that Phytomastigophora can synthesize vitamins of the "B complex." Among the higher Protozoa, on the other hand, synthesis of thiamin has been reported so far only for *Acanthamoeba castellanii* (Lwoff, 1938) and for two ciliates, *Tetrahymena geleii* and *T. vorax* (Kidder and Dewey, 1942). In each of these three cases the conclusion is based upon a premise which seems open to question—an unproven assumption that the basal medium was free from thiamin. Other investigations seem to have demonstrated that certain ciliates—*Colpidium striatum* (Elliott, 1937, 1939), *Glaucoma piriformis* (Lwoff and Lwoff, 1937, 1938; Hall, 1944), *Colpidium campylum* (Hall, 1940, 1942), *Colpoda duodenaria* (Tatum, Garnjobst and Taylor, 1942)—require thiamin from exogenous sources. Such a requirement parallels that of the higher animals and, with evidence from other sources, suggests that the higher Protozoa are more specialized than the plant-like flagellates with respect to vitamin requirements. Consequently, the possible synthesis of thiamin by ciliates is a question of some interest.

In attempts to develop a suitable medium for the study of vitamin requirements in *Glaucoma piriformis*, the writers have tried various protein preparations including a "vitamin-free casein" described as free from water-soluble and fat-soluble vitamins on the basis of tests with white rats. This type of casein was used previously by Dewey (1941) and by Kidder and Dewey (1942) in their work with ciliates. Kidder and Dewey concluded that *Tetrahymena geleii* and *T. vorax*, grown in a solution of this casein, could synthesize thiamin in the presence of a certain supplement ("Factor S"). Our experience with this "vitamin-free casein" forced us to consider the possible synthesis of thiamin by *Glaucoma piriformis*.

Material and Methods

The strain of *Glaucoma piriformis* was the one used by Hall and Shottenfeld (1941) and by Hall (1941, 1944). The ciliates were grown in the following basal media:

(1) Casein medium:

"Vitamin-free casein" (Casein-Harris)	10.0 gm.
Distilled water	1.0 liter

(2) Salted casein medium:

"Vitamin-free casein" (Casein-Harris)	10.0 gm.
$MgSO_4 \cdot 7H_2O$	0.2 gm.
K_2HPO_4	0.2 gm.
$CaCl_2 \cdot 2H_2O$	0.1 gm.
$FeCl_3 \cdot 6H_2O$	0.0025 gm.
Distilled water	1.0 liter

To this solution were added, each in a concentration of 1×10^{-7} gm. per cc., $MnCl_2 \cdot 4H_2O$ and $ZnCl_2$.

(3) Dethiaminized casein medium: A 2 per cent solution of Casein-Harris at a pH above 9.6 was heated in the autoclave for an hour at 122° C. According to previous experience (Elliott, 1939; Hall, 1942) such treatment should reduce the thiamin concentration below that required for growth of the ciliates. After treatment, the casein solution was diluted with salt solution and adjusted to pH 6.9 so as to produce a dethiaminized medium, homologous with the salted casein medium just described.

Each medium, with or without an added growth-factor as desired, was transferred to 16 mm. Pyrex culture tubes plugged with bleached non-absorbent cotton (Johnson and Johnson), and then sterilized in the autoclave at 122° C. for 20 minutes. Distilled water was redistilled in Pyrex glass before use. All inorganic salts were "analyzed reagent quality" (Mallinckrodt). Thiamin hydrochloride (Merck's Betabion), 2-methyl-5-ethoxymethyl-6-amino-pyrimidine and 4-methyl-5-B-hydroxyethyl-thiazole were tested in the basal media as described below. The preparations of thiazole and pyrimidine were obtained through the kindness of Dr. D. F. Robertson of Merck and Company.

In tracing growth of populations and growth in serial transfers, cultures were killed by heat (75–80° C.) before counting. Otherwise, our method of counting was essentially the same as that described previously (Hall, Johnson and Loefer, 1935).

Growth in Casein and Salted Casein Media

These two basal media, with and without added thiamin, were used for serial transfers. Thiamin (1×10^{-6} gm. per cc.) was added to a portion of each medium; the remaining portions served as controls. For transfers 1–3 each medium was adjusted to pH 6.7 before sterilization. At this pH solution of the casein was incomplete and the residue was discarded by decanting. Somewhat better results were obtained in transfer 4 by adjusting the medium to pH 6.9. In subsequent transfers the preliminary pH has been varied from 6.9 to 7.2. In transfer 1, all cultures were inoculated from a stock flask culture of *G. piriformis* in casein-peptone medium, and then incubated at 24 ± 0.2° C. Transfers 2, 3 and 4 were inoculated serially from the preceding transfers after 10, 14 and 14 days, respectively; subsequent transfers, after 1–4 weeks of incubation.

Growth of populations in transfers 1–4 is traced in Figure 1; in addition, transfer 11 of the strain in salted casein medium is described in Figure 3. In transfer 1, all the tubes contained a significant quantity of casein-peptone (1 : 2,000) from the stock culture; in the fourth transfer, serial dilution alone would have reduced the

peptone to not more than 1:2,000,000. After the first transfer, *G. piriformis* failed to grow in casein medium, either with or without added thiamin. In the salted media, however, growth occurred in serial transfers. The original strain without added thiamin has been maintained for 14 months (28 transfers), with the observed densities averaging a little more than 40,000 ciliates per cc. A second strain, started more recently from a stock in casein-peptone medium, has been carried for six months (14 transfers). Two subinoculations from the original salted-casein strain and two more direct inoculations from peptone stock cultures have failed to establish a strain in the unsalted casein medium. Accordingly, it

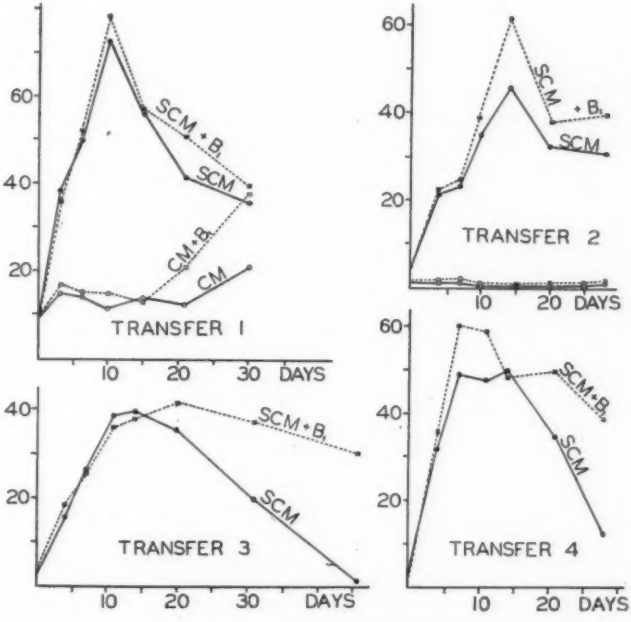

FIGURE 1. Growth of *Glaucoma piriformis* in casein medium (CM) and in salted casein medium (SCM); B_1, thiamin (1×10^{-6} gm. per cc.). Ordinate, thousands of ciliates per cubic centimeter. Populations are traced in point-to-point curves.

seems that Casein-Harris and a mixture of certain salts constitute a satisfactory culture medium for *G. piriformis*, whereas a one per cent solution of this casein without added salts is inadequate for growth. The mineral requirements of *G. piriformis* are still being investigated and will be considered more specifically elsewhere.

These series were planned to determine the effects of added thiamin, since this vitamin had increased population yield in certain other media (Hall and Shottenfeld, 1941; Hall, 1944) and was known to be essential for growth of *Glaucoma piriformis* (Lwoff and Lwoff, 1938) and certain other ciliates (Elliott, 1937, 1939;

Hall, 1940, 1942; Tatum, Garnjobst and Taylor, 1942). Somewhat surprisingly, added thiamin exerted little influence on maximal density, in distinct contrast to the effects of this vitamin when added to dethiaminized media (Hall, 1942) and depleted media (Hall, 1944). In spite of the comparatively slight effect on maximal density, thiamin did decrease the rate of death following the phase of maximal density—a decrease such as that observed under similar conditions in other media (Hall and Shottenfeld, 1941; Hall, 1944) known to contain thiamin, and likewise in cultures of *Tetrahymena geleii* (Johnson and Baker, 1943). Accordingly, on the basis of previous experience, the effect of thiamin in salted casein medium was just what would be expected in a medium already containing enough of this vitamin

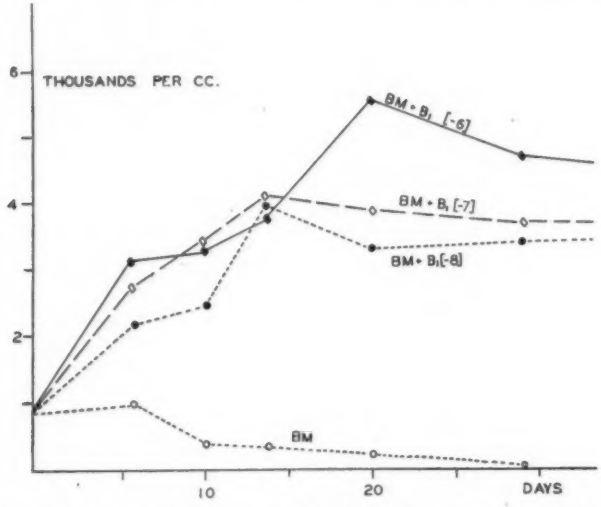

FIGURE 2. Growth of *G. piriformis* in dethiaminized casein medium. BM, dethiaminized medium; B_1 (—6), B_1 (—7) and B_1 (—8), thiamin in concentrations of 1×10^{-6}, 1×10^{-7} and 1×10^{-8} gm. per cc., respectively. The last point on each curve, at 70 days of incubation, is not included in the figure. All four series were started from one flask of dethiaminized medium seeded from a stock culture in salted casein medium (the original strain at transfer 9). The initial count was 850 ciliates per cc.; the initial pH, 6.9.

to support growth of *G. piriformis* to approximately maximal density, as defined by factors other than the supply of thiamin. In other words, there is no evidence that the available concentration of thiamin was a factor appreciably limiting the density attainable in salted casein medium. Hence, it seemed likely that Casein-Harris contains enough thiamin to support moderate growth of *G. piriformis* and that growth in the plain casein medium was limited by a mineral deficiency.

On the other hand, synthesis of thiamin has been attributed to *Tetrahymena geleii* and *T. vorax* grown in a medium (1.0 per cent solution of Casein-Harris with certain supplements) said to be "free of thiamin or its intermediates" (Kidder and Dewey, 1942). If Casein-Harris contains no thiamin, then *G. piriformis* either does not require thiamin or else synthesizes the vitamin from substances in our

salted casein medium. Although the observed effect of added thiamin would seem rather strange if *G. piriformis* is able to synthesize all the thiamin it needs, the possible thiamin requirements of this ciliate were tested with dethiaminized medium.

Growth in Dethiaminized Medium

The effects of adding thiamin to dethiaminized medium were determined by the serial transfer technique and by tracing populations in the first transfer. The method of inoculation was such that relatively fresh casein from the stock culture was reduced to less than 1 : 7,500 in the cultures of transfer 1. A control series was started in the basal medium, while three other series received thiamin in the indicated concentrations (Fig. 2).

In the first transfer (Fig. 2) no growth occurred in the basal medium and no active ciliates were seen in this medium after four weeks of incubation. With added thiamin, however, the populations reached 4,000–5,500, approximately, the highest density being obtained with the highest concentration of thiamin. At the end of 10 weeks cultures with thiamin still contained 2,500–3,500 ciliates per cc.

Subsequently, the ciliates have been grown in serial transfers (Series II, Table I) for nine months (15 transfers) in dethiaminized medium with added thiamin (1×10^{-6} gm. per cc.). The series with lower concentrations of thiamin were discontinued after the first transfer. The strain in the basal medium failed to survive beyond the fourth transfer. In a later attempt to establish a subsidiary strain in dethiaminized medium, series III (Table I) was started from series II at transfer 4. In this case also, the ciliates failed to survive after the fourth transfer.

Table I

Growth of Glaucoma piriformis *in dethiaminized casein medium*

Transfer	Series I, basal medium	Series II, basal + thiamin	Series III, basal medium
1	++	++++	
2	+	+++	
3	+	+++	
4	±	+++	
5	0	+++	+++
6	0	+++	+
7		+++	±
8		+++	±
9		+++	0
10		+++	0
11		+++	
12		+++	
13		+++	
14		+++	
15		+++	

++++, more than 2,500 ciliates per cc.
+++, 1,100–2,000 ciliates per cc.
++, 500–1,000 ciliates per cc.
+, less than 500 ciliates per cc.
±, very few ciliates, viability test positive.
0, no ciliates seen, viability test negative.

These observations indicate that *G. piriformis* requires thiamin for growth and are in agreement with the findings of Lwoff and Lwoff (1938) for this species. The failure of dethiaminized casein medium to support growth of *G. piriformis* presumably indicates that thiamin, present in adequate concentration in Casein-Harris, was largely destroyed by heating the casein in alkaline solution. Such an interpretation of similar results forms the basis for conclusions that various Trypanosomidae and certain ciliates—*Colpidium striatum* (Elliott, 1937, 1939) and *Glaucoma piriformis* (Lwoff and Lwoff, 1938)—require thiamin for growth.

The continued growth of *G. piriformis* in salted casein medium might be explained on the basis that synthesis of thiamin is catalyzed by one or more of the added salts. On the other hand, since *G. piriformis* did not grow in unsalted casein solution with added thiamin, it seems likely that the casein solution is deficient in minerals essential to growth and hence will not support growth even when thiamin is added. However, it is conceivable that any salts, which might be essential as catalysts in biosynthesis of thiamin, could be required also for growth of *G. piriformis* and for utilization of thiamin by this ciliate. Hence, in our experiments with dethiaminized medium the usual salt mixture was added *after* heating the casein in alkaline solution. In this way, unfavorable changes were limited to constituents of the casein solution. It will be noted (Fig. 2; also, Table I) that the addition of thiamin to dethiaminized medium did not restore the population to a normal level, although insuring growth in serial transfers. This limited effect of thiamin must be attributed to some change in the casein itself during dethiaminization, and not to a deficiency in any salts which may be involved in utilization or synthesis of thiamin.

A clue to the nature of such additional changes in dethiaminized casein was obtained by the addition of both riboflavin (1×10^{-7} gm. per cc.) and thiamin (1×10^{-6}) to dethiaminized medium. The density of population was raised to a level approaching that in fresh salted casein medium—in serial transfers, the density has ranged from approximately 20,000 to 35,000 per cc. after different periods of incubation. This relation of riboflavin to growth of *G. piriformis* has been observed also in depleted media and will be discussed elsewhere (Hall, 1944), but it may be pointed out that the present results suggest the presence of both riboflavin and thiamin in Casein-Harris.

Effects of Thiazole and Pyrimidine

The results obtained with dethiaminized medium indicated that *G. piriformis* requires thiamin for growth and that an adequate supply of riboflavin also is needed for normal population yields. If Casein-Harris actually contains neither thiamin nor its components, as implied by Kidder and Dewey (1942), the ability of *G. piriformis* to synthesize thiamin would have to be fairly extensive in order to produce the growth curves described in Figures 1 and 3. Such a ciliate, if it is to synthesize thiamin, should be able to utilize the thiazole and pyrimidine components in biosynthesis, since this appears to be true for microorganisms known to synthesize this vitamin. Accordingly, addition of thiazole and pyrimidine to salted casein medium might be expected to produce a beneficial effect comparable to that of added thiamin.

In considering this possibility, the growth of *G. piriformis* in salted casein

medium was compared with that in media containing: (a) the thiazole and pyrimidine components of thiamin; and (b) thiamin. The results (Fig. 3) show no beneficial effects of added thiazole and pyrimidine, since the population receiving these supplements did not differ significantly from that in the basal medium during 52 days of incubation. Added thiamin, on the other hand, produced the usual lower rate of death after the phase of maximal density. We are forced to conclude that *G. piriformis* did not synthesize thiamin from thiazole and pyrimidine in any significant quantity, and that the relatively rapid death of the ciliates in the basal medium and with the added components of thiamin was the result of a thiamin deficiency.

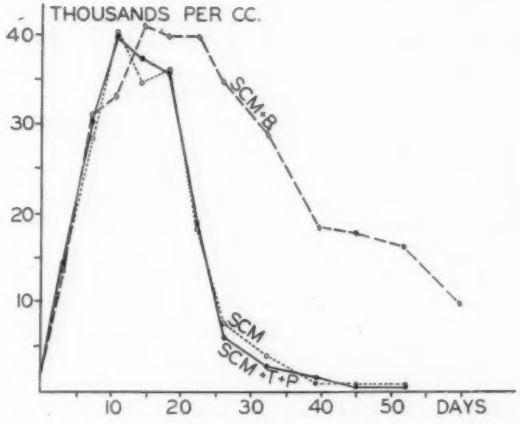

FIGURE 3. Growth of *G. piriformis* in salted casein medium. SCM, salted casein medium; B_1, thiamin (1×10^{-6} gm. per cc.); P, 2-methyl-5-ethoxymethyl-6-amino-pyrimidine (1×10^{-6} gm. per cc.); T, 4-methyl-5-B-hydroxyethyl-thiazole (1×10^{-6} gm. per cc.). All three series were inoculated from a flask of the basal medium seeded from a stock in salted casein medium (the original strain at transfer 10). The initial count was 173 ciliates per cc.; the pH, 6.8.

By means of the serial-transfer technique, the effects of added thiazole and pyrimidine were determined also in dethiaminized casein medium. Three series were started—one with the basal medium, a second with added thiazole and pyrimidine (concentrations as in Fig. 3), and the last with added thiamin (1×10^{-6} gm. per cc.). The initial pH in transfer 1 was 6.9, and all three series were inoculated from transfer 10 of series II (Table I). The series with added thiamin was discontinued after the sixth transfer, growth having approximated very closely that in corresponding transfers of series II (Table I). In the other two series, very little growth occurred in the fourth transfer, no normal ciliates were seen in the fifth, and viability tests (casein-peptone medium) of the sixth transfer cultures were negative. No beneficial effects of added thiazole and pyrimidine were detected, and these substances obviously could not be substituted for thiamin in dethiaminized casein medium.

Discussion

The results obtained with *Glaucoma piriformis* afford no basis for concluding that this ciliate synthesizes thiamin under the conditions of our experiments. Yet our basic findings for this species coincide with those reported for *Tetrahymena geleii* and *T. vorax* (Kidder and Dewey, 1942). All three of these ciliates failed to grow in a one per cent solution of Casein-Harris. Furthermore, the addition of thiamin did not enable the organisms to grow in serial transfers. Such a casein solution obviously is an inadequate medium for these ciliates, and the failure of added thiamin to correct the deficiency would seem to demonstrate that this casein preparation lacks some other substance or substances essential to growth.

Kidder and Dewey (1942, table 6) found that a combination of casein solution and a "heat and alkali treated water extract of alfalfa meal" supported growth of their ciliates. These results form the basis for their conclusion: "Two of the ciliates (*Tetrahymena geleii* and *T. vorax*) were found to be able to synthesize thiamin if a substance from leaves, Factor S, was added to a medium free of thiamin or its intermediates." Such an assumption does not explain the failure of *T. geleii* and *T. vorax* to grow in casein solution with added thiamin. In other words, if growth is possible when an added catalyst enables the ciliates to synthesize thiamin from an otherwise satisfactory medium, then growth should occur also when exogenous thiamin is supplied.

The writers have shown that the addition of certain salts to casein solution (Casein-Harris, one per cent) insures growth of *G. piriformis* in serial transfers. In view of these results, it might be assumed that synthesis of thiamin is catalyzed by certain of the added salts. However, there would remain the facts that *G. piriformis* did not grow in simple casein solution with added thiamin, and that these same salts did not enable *G. piriformis* to synthesize thiamin in dethiaminized casein medium. Consequently, it seems entirely probable that the casein solution is deficient in essential salts and without such supplements will not support growth even when thiamin is added.

Populations of *G. piriformis* in dethiaminized casein medium and in depleted media (Hall, 1944)—media which are apparently unsuitable for synthesis of thiamin by this ciliate—have been found to benefit greatly from added thiamin. In fact, there has been no growth in certain instances without added thiamin. Thus it is obvious that growth of *G. piriformis* depends upon a readily available supply of thiamin. If it is true that our salted casein medium contains no thiamin, then added thiamin should exert an immediate influence on growth of populations. Instead, the densities in the basal medium and in that containing thiamin as a supplement were not significantly different until after the populations reached maximal density (Figs. 1, 3). Such results would not be expected if Casein-Harris were actually free from thiamin.

The conclusion of Kidder and Dewey, that *T. geleii* and *T. vorax* synthesize thiamin when supplied with "Factor S," is based primarily upon the assumption that Casein-Harris contains no thiamin. It must be pointed out, however, that this casein is biologically standardized with the white rat as the test organism and *on that basis* is described as vitamin-free. Kidder and Dewey have presented no additional evidence. Actually, Casein-Harris might well contain enough thiamin for moderate growth of *G. piriformis,* since nicotinic acid (3.6 micrograms per gram)

has been demonstrated in a "vitamin-free casein" (Noll and Jensen, 1941). Furthermore, it has been reported (Halliday and Duel, 1941) that about 40 per cent of the thiamin in milk is in non-dialyzable form, probably combined with proteins in a complex which can be broken down by such a proteinase as papain, a type of enzyme produced by *G. piriformis* (Lawrie, 1937). Since Casein-Harris is washed by reprecipitation and extraction with dilute acids, the treatment might leave a little residual thiamin—enough to support growth of *G. piriformis* as observed in serial transfers.

Accordingly, the most probable interpretation of our data involves the following conclusions: (1) Casein-Harris does not contain an adequate supply of certain minerals essential to growth of *G. piriformis*. (2) The effects of thiamin, added to our salted casein medium, are similar to those produced in other media known to contain thiamin—there was little if any increase in population, although the rate of death following the phase of maximal density was lower than in the control medium. (3) The effects of thiamin, added to salted casein medium, are decidedly different from those produced in dethiaminized and depleted media, in which the density of population is increased promptly. (4) Casein-Harris apparently contains enough thiamin to support growth of *G. piriformis* in serial transfers. (5) The failure of added thiazole and pyrimidine to influence growth, either in salted casein medium or in dethiaminized casein medium, affords no evidence that *G. piriformis* utilizes these components in the synthesis of thiamin. (6) Our findings supply no evidence that *G. piriformis* can synthesize thiamin in casein medium, in salted casein medium, or in dethiaminized casein medium.

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THE MECHANISM OF EXTENSION IN THE LEGS OF SPIDERS [1]

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Introduction

Prior to 1909 morphologists studying the leg musculature of spiders unanimously considered movement in each joint to be controlled by antagonistic muscles. The femoro-patellar and tibio-metatarsal joints (the two most important flexor-extensor joints in the leg), however, are not supplied with such paired muscles, since there are no extensor muscles (Petrunkevitch, 1909). The alacrity of extension in these joints, therefore, presents the singular problem of accounting for these "muscle-less" extensor movements.

The assumption has been made by Petrunkevitch (1909, 1916, personal communication 1941) that "the function of extension has been taken over entirely by the elastic interarticular membrane." Except for this single suggestion by Petrunkevitch, no mechanism has been forwarded to account for these forceful and rapid extensor movements. It has seemed worthwhile, therefore, to investigate this problem.

The spiders used in the present study have included the tarantulas, *Dugesiella californica, Delopelma helluo,* and *Aphonopelma cryptethus* representing the suborder Mygalomorphae, and *Agelena naevia* (the grass spider), *Miranda aurantia* (the golden garden spider), *Latrodectus mactans* (the black widow), and *Aranea cavicata* (large orb-weaver) representing the suborder Dipneumonomorphae. All of the specimens were collected in or near Pasadena, California.

With respect to the morphological and functional relationships studied in the present investigation, no significant differences between the species were found.

Results and Discussion

A. *The possibility of extension by muscular contraction*

That muscular contraction is not the extensor mechanism is obvious. In agreement with Petrunkevitch it has not been possible to find any trace of extensor muscles (see below) in the two joints under consideration. Furthermore, the axis in each of these joints is located dorsally in such a way as to preclude attachment of extensor muscles. At these joints the appendages are flattened over the dorsum in the articular region, and are joined by very narrow dorsal interarticular membranes. During flexion or extension these membranes serve as simple hinges. With this extreme dorsal location of the axis there is no conceivable way in which

[1] This investigation formed a portion of a thesis submitted to the Faculty of the Graduate School of California Institute of Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in June, 1943.

internal muscular attachments could be made whereby muscular contraction would produce extension of the joint, and it is certain that no tendons pass externally over the hinge. Therefore, the theory of muscular extension must be discarded.

B. *The possibility of extension by the interarticular membrane*

There are two interarticular membranes (Fig. 1 dm, vm), one dorsal, the other ventral. Of these the dorsal one is very short. Petrunkevitch (1909) claimed for this dorsal membrane the un-assisted function of extension, but examination of his paper has not given the experimental basis upon which he based this conclusion.

Were the elasticity of this membrane the mechanism involved in extension it would seem imperative that the spider assume a position of complete extension in death. This is not the customary position taken; spiders nearly always die with their legs completely and permanently flexed. In fact, so characteristic is the flexed position in death that when startled a number of spiders assume the position as a protective reflex (Robertson, 1904).

It might be reasoned that this position of extreme flexion is due to an irreversible contracture in the muscles. That such a mechanism is not responsible for this flexed position was shown by the following experiment: A leg was removed at the trochanter and the chitin covering the ventral half of the femur was removed, due care being taken to leave both the ventral and dorsal interarticular membranes intact. The muscles in the femur were transected as distally as possible, and those in the patella were loosened at their origin. The same procedure was carried out in the tibio-metatarsal joint, and in neither case did extension occur. In fact, the joint remained in precisely that position in which it was manually placed. The legs from spiders of a number of different genera were used, and in all instances these results were observed. It is obvious, therefore, that the membranes themselves have little if any elasticity. This would certainly appear to render untenable any theory basing either extension or flexion on an inherent elasticity of the interarticular membranes.

C. *The effects of electrical stimulation*

In the following experiments the leg was removed at the trochanter and was anchored at the femur to the stage of a binocular dissecting microscope with plasticine. In most instances the tip of the tarsus was attached to a very lightly weighted heart lever by a length of silk thread, and the movements were recorded on a smoked kymograph drum. Stimulation was produced with micromanipulated platinum electrodes using a DuBois-Reymond inductorium.

Various ways of stimulating were used in an effort to produce extensor movements. The nerve was prepared in the femur and was stimulated either in its entirety or in bundles after splitting the nerve with a fine needle. In some instances the tendons of the flexor muscles were cut prior to stimulation of the nerve. In all instances, however, stimulation with currents of ordinary intensity resulted only in flexor contractions if any response was obtained. This supports the finding that no extensor muscles are present.

The functions of the respective muscles in the segments of the leg distal to and including the femur have been investigated by selectively stimulating each of the

muscles. Most of these studies were made on the walking legs of tarantulas, although some observations have been made on the legs of *Agelena naevia, Latrodectus mactans,* and *Miranda aurantia.* No differences between the species were found.

The leg was opened with a pair of small scissors and the chitin from the side opposite the muscle to be examined was removed with a pair of fine forceps. Small platinum electrodes mounted in Zeiss micromanipulators were then applied to the muscle with the aid of a dissecting microscope. Contraction of the muscle was ob-

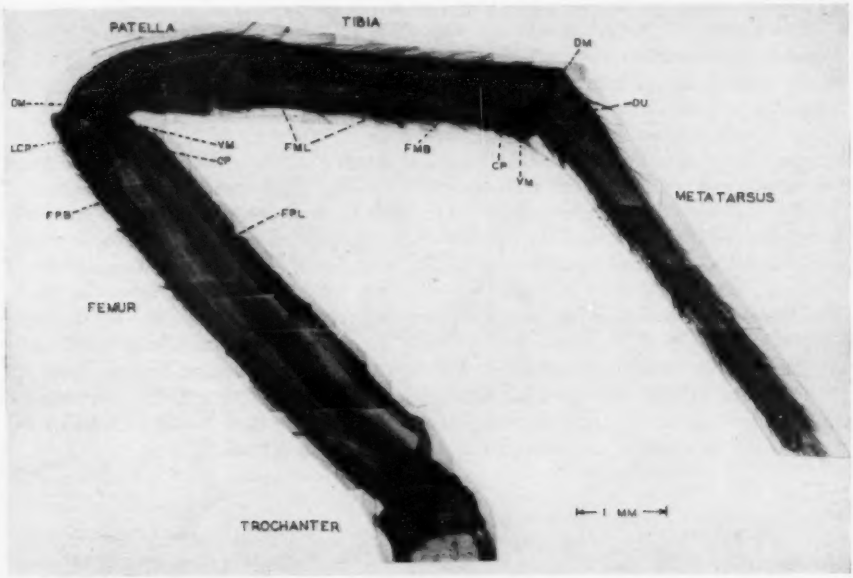

FIGURE 1. Composite photomicrograph of the mid-sagittal section of a walking leg of *Miranda aurantia.* CP = chitinous plate, DM = dorsal interarticular membrane, DU = depressor unguium muscle, FMB = flexor metatarsi bilobatus, FML = flexor metatarsi longus, FPB = flexor patellae bilobatus, FPL = flexor patellae longus, LCP = levator of chitinous plate, VM = ventral interarticular membrane.

served under the microscope to prove the selectivity of stimulation and movements of the parts of the leg were observed visually.

The muscles of the femur showed the following functions: The *flexor patellae bilobatus* (Fig. 1 fpb) produced strong flexion of the femoro-patellar joint. As Petrunkevitch (1909) and Brown (1939) reported, this bitendonous muscle lies laterally and dorsally in the femur, arising from the dorsal femoral surface and inserting directly on the proximal lip of the patella. The *flexor patellae longus* (Fig. 1 fpl), arising from the ventro-lateral lip of the trochanter and from the ventral proximal portion of the femur, extends ventrally and medially the full length of the femur, inserting, not on the proximal lip of the patella as has been described

(Petrunkevitch, 1909), but on the proximal margin of a chitinous horseshoe-shaped plate to whose distal margin are attached the proximal and distal portions of the ventral interarticular membrane (Fig. 3 cp). Stimulation of either of the bellies of this muscle likewise resulted in a flexor movement, although of considerably less intensity than that of the *flexor patellae bilobatus*.

An homologous picture was found in the tibia; the strongest flexor movement was obtained from stimulation of the *flexor metatarsi bilobatus* (Fig. 1 fmb), while the movements resulting from stimulation of the *flexor metatarsi longus* (Fig. 1 fml), although also flexor in nature, were much weaker than those of the former muscle.

Stimulation of the *depressor unguium* (Fig. 1 du) in the tibia, of the *levator unguium* in the metatarsus, and of the two main muscles in the patella gave the expected results, i.e., depression and elevation of the claw and the respective lateral movements of the tibia.

It has thus been possible to confirm most of the morphological findings of Petrunkevitch (1909) and Brown (1939), and to establish at the same time a clear functional proof of the specific activities of most of the muscles in the spider leg.

It was observed that "closed-leg" preparations, i.e., those in which the electrodes were inserted through the chitin of the femur, gave extensor movements of the two joints under consideration when stimulated very strongly for several seconds (coil distance = 3 to 1½ cm.). Upon removing the tip of the tarsus, however, such stimulation resulted in a much less pronounced extensor movement and was accompanied by the appearance of a growing droplet of blood at the severed end. Sealing the tip with plasticine resulted in a restoration of extensor movements of the original height. This stimulation was of such intensity that bubbles of hydrogen and oxygen appeared on the electrodes, and it is likely that the accumulation of gases was instrumental in producing the extensor movements.

D. *The possibility of an hydraulic extensor mechanism*

If the liberation of gases by electrolysis is sufficient to so increase the internal pressure of the leg that a straightening of the appendage occurs, it would seem quite feasible to consider that increases in the fluid pressure within the leg could result in extension. That such an increase does result in an extensor movement can be shown by gently squeezing an isolated leg. Extension occurs at the joints with a ballooning of the ventral interarticular membrane. Gaubert (1892) reported the phenomenon and considered it indicative of an extensor mechanism accessory to the "extensor muscle" which he firmly believed to exist.

Injection of fluid with a hypodermic syringe into any part of the leg, whether isolated or on the living or dead animal, is accompanied by extension of all parts distal and proximal to the point of injection. Injection into the abdomen or cephalothorax also produces extension of the appendages. The pressure necessary for producing this effect is small, a very light touch on the plunger of the syringe being sufficient to produce extension. Petrunkevitch (1910), although not mentioning the possibility of an hydraulic extensor mechanism, observed the above phenomenon, and used it as an indication of the completeness of injection while studying the circulatory system of spiders. He said, "Die Injektion musz so lange fortgesetzt werden, bis alle Beine straff auseinandergespreizt bleiben und die

Stacheln an ihnen nahezu senkrecht abstehen." Ten Cate (1931) showed that in Celaenia cutting off the abdomen resulted in stretching of the legs, but once the abdomen had been removed, the animal fell down as in death, i.e., the legs were flexed and no further extensor movements occurred. It would appear obvious that the constriction during the cutting process would momentarily increase the pressure giving rise to the extensor response.

That such hydraulic extension can be brought about mechanically does not give sufficient grounds, however, for assuming that the same mechanism is involved in the normal extensor movements of the live animal. The following experiments were therefore undertaken in an effort to see whether or not any similar pressure changes normally occur.

In the intact tarantula cutting off the tip of a leg produces a condition in which that leg is not extended upon stimulation of the animal in a manner which normally results in extension of the appendages, i.e., holding the animal in an inverted position. Sealing the tip restores the extensor function. The same effect has been obtained by opening the chitin of the femur in the intact spider. In such an animal pressure applied distal to the opening results in extension of the two joints, but pressure applied proximal to the opening results only in a marked effusion of fluid from the hole. Moreover, so long as the hole is open, the animal moves around with the operated leg in a flexed position, whereas the unoperated legs show normal extensor activity. The same result was obtained in *Agelena naevia*.

Internal fluid pressure. Spiders always appear to be maintaining a high internal pressure within their bodies and appendages, as is seen by the fact that even a small perforation of the chitin of any living spider results in a great loss of blood whether the hole be in the abdomen, cephalothorax or appendage. In order to investigate the effect of lowering this internal fluid pressure, a number of tarantulas were dehydrated by keeping them for several weeks without water. They showed, prior to death, a condition in which walking was not accompanied by extension of either the femoro-patellar or tibio-metatarsal joints, and the spiders walked with their legs in a more or less markedly flexed position. In such an animal (tarantula) injection of 2 ml. of a salt solution [2] was accompanied by a period in which the two above-mentioned joints were extended as readily as in the normal animal. It should be recalled, however, that any perforation is accompanied by a considerable loss of blood, and upon removal of the injection needle from the abdomen, the animal bled profusely and in a short time again lost the ability to extend the legs. A second injection of physiological solution restored the normal condition, and when the hole was closed with collodion the animal ran about the cage in a more normal way, making use of its extensor mechanism.

The blood volume of the spider has also been lowered experimentally by withdrawing an appreciable amount of blood, using a small hypodermic needle, whereupon extension became markedly slower and less effective. Replacing the withdrawn blood with physiological solution gave a return of quick extensor movements of normal magnitude.

[2] Frog Ringers and several other salt solutions were unsatisfactory. Since these solutions had a molar concentration considerably lower than that of the physiological solution for crayfish described by van Harreveld (1936), the latter fluid was tried. It proved to be quite satisfactory, the animal remaining alive for somewhat longer than one hour following injection.

It has been pointed out that spiders characteristically die with their legs in a strongly flexed position. An interesting exception to this rule was observed in the case of a male tarantula which fell into its dish of drinking water and drowned. In this animal the legs were almost fully extended. Furthermore, the slightest pressure applied to the abdomen was sufficient not only to extend the legs maximally, but to raise the spines as well. It is evident that the spider in this case had imbibed water to such an extent that an abnormally high fluid pressure was present.

From the foregoing experiments the conclusion can be drawn that the internal fluid pressure of the spider must be kept high if maximum extensor activity is to be maintained.

Cardiac pulsations in the leg. Plateau (1887) and Gaubert (1892) observed that when spiders are suspended so that one or more of the legs is without a point of rest, those legs successively extend and flex through a small arc synchronously with the systolic and diastolic phases of the heart beat. Willem and Bastert (1918) and Willem (1920) made use of this movement of the tarsi to record changes in the cardiac rhythm when the spider was stimulated by various means, e.g., shining a bright light in the eyes of the animal. They showed that these flexor-extensor movements of the tarsi were more rapid during systole (extension) than during diastole (flexion), and that these movements could thus be considered to be a true picture of the sequences of cardiac pulsation. It has been possible to confirm this synchronization of rhythmic leg movements with the heart beat in the crab-spider, *Misumena aleatoria,* in which the movement of the dorsal chitin of the abdomen with each heart beat is conspicuous. Only the femoro-patellar and tibio-metatarsal joints showed the above phenomenon, and the ventral thin-walled sac at each of these joints showed ballooning with the extensor phase of each movement.

The experimental evidence which has been presented indicates that extension in spiders is intimately associated with the internal hydraulic pressure of the leg, which is in turn associated with the blood volume within the appendage. It also seems likely, although not certain, that the claws are spread apart by an increase of hydrostatic pressure. They are pulled together, however, by muscular action, and elevated and depressed by contraction of the appropriate muscles. For the complex movements of the male copulatory apparatus Osterloh (1922) has presented evidence that an hydraulic mechanism is present.

The details of the hydraulic mechanism are not obvious. An increase in the internal fluid pressure could be obtained in several ways. (1) The venous return pathway could be partially blocked, thereby permitting a greater volume of blood to accumulate in the appendage. Some mechanism of this sort must be present, or it becomes difficult to account for the fact that fluid is not forced out of the proximal end of an isolated leg upon compression of the femur. (2) The arterial flow of blood into the leg could be diverted from its normal path so as to produce an effective extensor thrust by ballooning the ventral interarticular membranes. A combination of these mechanisms may be involved.

There are, however, certain peculiarities of structure which have been observed in the two joints in which this type of extensor control is found. These should be considered as to possible roles in extension.

Structural peculiarities. From the ventral tibio-metatarsal and femoro-patellar interarticular membranes arise chitinous plates (one in each joint) which extend

proximally for a short distance. These plates are flattened dorso-ventrally and are bowed proximad. The plate in the femoropatellar joint was described by Gaubert (1892) but its presence has been consistently ignored by subsequent investigators, and the homologous structure in the tibio-metatarsal joint has apparently never been reported. From the proximal margin of these plates arise the tendonous strands whereby the *flexor metatarsi longi* and *flexor patellae longi* are respectively inserted. Moreover, a few isolated muscle fibers, arising from the distal dorsal wall of the femur and inserting on the dorsal surface of the chitinous plate (Fig. 2 lcp), seem to be uniformly present in sagittally cut preparations.[3] It was first thought that these fibers might be artifacts resulting from the cutting procedure, but care was taken to make the cut from different directions with respect to

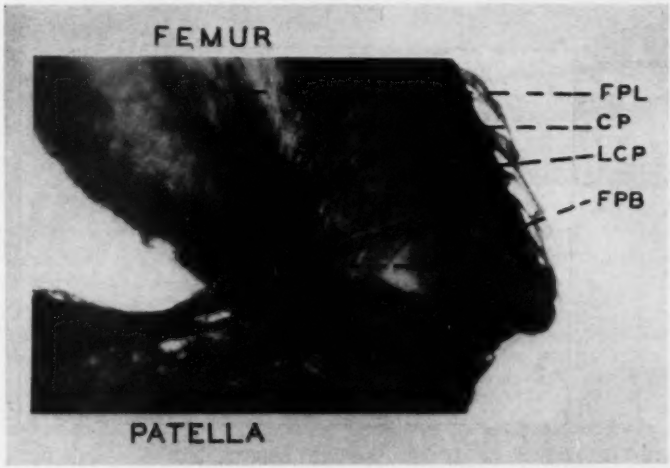

FIGURE 2. Photomicrograph of the femoro-patellar joint of *Miranda aurantia*. (Mid-sagittal section.) CP = chitinous plate, FPB = flexor patellae bilobatus, FPL = flexor patellae longus, LCP = levator of chitinous plate.

the femoro-patellar joint, and in all cases these fibers were found to lie in almost identically the same position on the dorso-ventral median line of the femur. It appears, therefore, that these few fibers form an heretofore undescribed muscle, the function of which is obviously elevation of the chitinous plate. It is probable that this group of fibers has been overlooked because they run in nearly the same direction as the more laterally located distal fibers of the *flexor patellae bilobatus*. Careful examination of Figure 2 will show, however, that these fibers of the large flexor muscle insert on the chitinous lip of the patella, not on the plate. The existence of

[3] The above sagittal preparations were made by first fixing the isolated leg in Petrunkevitch's (1933) copper-tri-nitro-phenol, dehydrating with graded alcohols and xylol, and imbedding in paraffin. The paraffin block was then trimmed under the dissecting microscope with a sharp scalpel until the mid-sagittal plane was reached. The excess paraffin was removed in xylol, the half-leg washed through several fresh xylol baths, and finally mounted in balsam.

an homologous group of fibers elevating the chitinous plate in the tibio-metatarsal joint is still uncertain.

The distribution of the arteries into the muscles and other tissues of the leg has never been adequately described. Causard (1896) figured the pedal arteries as simple uninterrupted tubes, one in each leg, emptying into the cavity of the leg through a single perforation in each segment except the tarsus where the artery ends as an open tube. Petrunkevitch (1910) stated that it is possible to trace the pedal arteries as far as the claw, but said nothing concerning any branching within the leg. Profuse branching of the main pedal artery occurs, however, as can be seen by injecting an animal with a colored latex medium.[4, 5] The branches arborize into the muscles in a manner quite similar to that described by Causard (1896) for the muscles of the cephalothorax (Fig. 3).

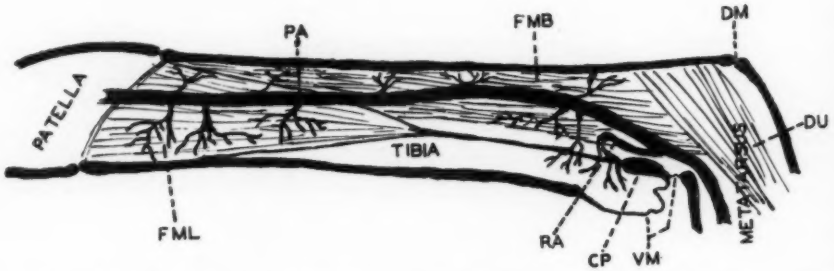

FIGURE 3. Diagram showing the vascularization in the tibia of a spider. CP = chitinous plate, DM = dorsal interarticular membrane, DU = depressor unguium, FMB = flexor metatarsi bilobatus, FML = flexor metatarsi longus, PA = main pedal artery, RA = reflected arterial branch, VM = ventral interarticular membrane.

With respect to a possible structural relation between the distribution of these vessels and the extensor function in the two joints under discussion, two peculiarities should be described. (1) Careful examination of the distribution of the main arterial trunk in the appendage shows that, although it runs somewhat dorsally through the major part of the femur and tibia, on approaching the respective joints, it dips ventrally and passes in close proximity over the chitinous plate. (2) In several preparations of the legs of tarantulas, a rather large branch has been found

[4] In order to obtain any degree of success with injection of the arterial system in the legs of spiders it was found necessary to remove the tips of the tarsi to provide an outlet for the increased pressure created by injection. The method of injection, therefore, was as follows: The spider was killed by placing it in a bottle containing a few drops of chloroform. The diluted alkaline latex solution was sucked up into the injection pipette, the tip of which had been drawn out into a rather fine capillary. The pipette was inserted through the dorsal wall of the abdomen, and the latex medium was forced into the animal with compressed air. Dilute HCl was then injected by hypodermic syringe, forcing the acid into the body and appendages at various places, thus setting the latex. It was found that when the legs were left intact, no injection fluid was able to penetrate the vessels of the appendages, but when the tips of the legs were removed, a most satisfactory injection of even the finest branches was obtained.

[5] The author wishes to express his appreciation to Mr. C. Blair Coursen of the General Biological Supply House, Inc., who kindly made available a sufficient supply of the latex injection medium.

to arise from the main pedal artery just proximal to the plate in the tibio-metatarsal joint. This branch is first reflected in a proximal direction for a short distance, whereupon it dips ventrally, arborizing laterally into the tissues surrounding the membranous sac bounded by the ventral interarticular membrane (Fig. 3 ra).

This distribution suggests that a mechanism for extension might involve the partial closing off of the main arterial flow, thus diverting a greater amount of blood into the region of the membranous sac, ballooning the latter structure and producing extension. Such a mechanism would have the distinct advantage of selectivity, either the tibio-metatarsal or femoro-patellar joint being independently extensible.

In order to examine the role which the plate may play in this function a study of its movement during flexion and extension of the leg has been made. Such motion can be watched through the thin transparent ventral interarticular membrane. The observation has been made that during flexion of the femoro-patellar joint the chitinous plate remains close to the ventral membrane. This is to be expected from the position of the attachments of the *flexor patellae longus* which, lying ventrally, pulls the plate in a horizontal plane. Because of the ballooning of the ventral interarticular membrane by the inflow of blood with each extensor movement it was not possible to determine whether the plate is pulled deeper during extension or whether this only appears to be so because of the ballooning effect. At any rate, one loses sight of the plate during extensor movements. That contraction of the fibers which lift the plate occurs during extension can only be assumed, therefore, and their designation as an auxiliary extensor mechanism must await further investigation.

Summary

The absence of extensor muscles in the legs of spiders presents the problem of accounting for the quick extensor movements characteristic of these animals. The theory that the "elasticity" of the interarticular membrane might be the means whereby this is accomplished has been shown to be untenable.

Experimental evidence has been presented showing that extension is intimately associated with changes in the volume of blood in the spider leg, i.e., with changes in the internal fluid pressure in the leg.

Certain structural peculiarities in the joints have been described, but the evidence is insufficient to permit evaluation of their possible roles in extension.

The author wishes to express his gratitude to Professors C. A. G. Wiersma and A. van Harreveld for their helpful suggestions during this investigation.

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LOCALIZATIONS OF ALKALINE AND ACID PHOSPHATASES IN THE EARLY EMBRYOGENESIS OF THE CHICK[1]

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Since Gomori's publication of a histochemical method for the demonstration of alkaline phosphatase, several studies of the distribution of alkaline phosphatase within tissues have appeared. Gomori himself (1941a) made a survey of the enzyme in a variety of adult mammalian organs; Kabat and Furth (1941) studied its occurrence in normal and tumorous tissues of mammals and chickens; Bourne (1943) investigated its presence in numerous tissues, giving special attention to the intra-cellular localizations; and Horowitz (1942) examined the interrelations of phosphatase and glycogen in fetal heads. Gomori (1941b) employed his somewhat less satisfactory technique for acid phosphatase in a study of acid phosphatase distribution in normal and tumorous tissues, and Wolf, Kabat and Newman (1943), after modifying the original technique to produce more regular results, used it in a study of acid phosphatases in the nervous system.

The present survey of the distribution of phosphatases in the early embryo of the chick was undertaken for two reasons. First it seemed likely that just as the studies cited above have aided in establishing correlations between phosphatase content and function in mature organs, so an embryological study might further elucidate the problem of the functional rôles of phosphatase by establishing correlations between phosphatase content and functional state in developing organs. Second, because of our increasing knowledge of the widespread importance of phosphate transfer in metabolism, it seemed worthwhile to examine the possibility that phosphatases play a part in the proliferative and differentiative processes of the early embryo.

Materials and Methods

These studies are based on White Leghorn embryos taken in stages from the unincubated blastoderm through the eighth day of incubation for all series of observations except those on the long bones of the hind limb, for which nine- and ten-day limbs were also used. At all stages, cold acetone was employed as fixative; after 2 to 24 hours in acetone, depending on size, the embryos were cleared in cedarwood oil and benzene, embedded in a paraffin-beeswax-bayberry wax mixture, and sectioned at 10 or 15 micra. Shrinkage of the cytoplasm occurred in many cells, but for gross histological examination chilled acetone proved an excellent fixative; for the preservation of alkaline as well as acid phosphatase it was superior to 85 per cent alcohol.

The histochemical method for the demonstration of alkaline phosphatase has been repeatedly described (Gomori, 1941a; Kabat and Furth, 1941; Bourne, 1943).

[1] Aided by a grant from the Rockefeller Foundation to Washington University.

The method was employed in these studies exactly as presented by Gomori, except that sodium glycerophosphate containing equal parts of the alpha and beta salts was used, since pure alpha glycerophosphate was not available; the pH was 9.3, and the time of incubation 1½ to 4 hours, for different specimens, at 38° C. Erythrosin was used as counterstain.

In the case of the acid phosphatase, Gomori's (1941b) formula for the incubating solution was altered to compensate for the precipitation of lead beta-glycerophosphate, as follows:

Acetate buffer at pH 4.8	2½	parts
5% lead nitrate	1	part
Distilled water	15	parts
2% Na glycerophosphate	3	parts

The ingredients should be mixed in the order given, and the solution be allowed to stand overnight in the refrigerator, since the precipitate develops slowly. The solution was filtered just before use, at which time any additional reagents were added. The pH is 5.1; the incubation time ranged from 5 to 16 hours, at 38°, since the concentration of acid phosphatase in most embryonic tissues is extremely low. In some cases at each stage 0.01 M $MnSO_4$ was added to the incubating solution in order to produce the stable black-staining precipitate described before (Moog, 1943b); and after it was discovered that ascorbic acid is a strong activator of acid phosphatase, series were also prepared with 0.01 M ascorbic acid added to the incubating solution. This phenomenon will be discussed in a later section.

After incubation the slides were rinsed in distilled water and dilute acetic acid, and stained by 2 minutes' exposure to light yellow ammonium sulfide, which results in the formation of brown or black lead sulfide. After thorough washing under the tap, the slides were dehydrated in the usual manner, counterstained with erythrosin, and mounted.

At all stages controls were made to check on the possible presence of preformed phosphates, by incubating slides in a solution from which glycerophosphate was omitted, and then staining them in the usual way. To a considerable extent also the alkaline and acid series served as controls for each other, since any tissue appearing negative to either enzyme could not contain preformed phosphate.

The stability of alkaline phosphatase in chick embryos has already been remarked on. The acid phosphatase is however troublesome to demonstrate with any regularity, so that material used for the study of this enzyme must be handled with great caution (cf. Moog, 1943a). The use of manganese improves the production of clearly visible precipitates in tissues low in acid phosphatase, but it does not control the considerable variations in activity found among individual embryos. Ascorbic acid, on the other hand, has proved valuable by reason of its ability to increase both the intensity and the uniformity of the normal deposits, especially in fresh preparations. Yet even with ascorbic acid variations continue to appear, and seem to reflect the extreme lability of the enzyme; for example, it will survive only a few days' storage in the refrigerator, even in paraffin, and it is quickly inactivated by temperatures above 60° C. The penetrating power of acetone was not a limiting factor, as in the tissues Wolf, Kabat and Newman (1943) used, for the deepest layers of the liver, even in eight-day embryos, gave more consistent results than some of the more superficial tissues.

Results

I. Alkaline Phosphatase

In the embryonic stages studied, alkaline phosphatase has been found present more generally in nuclei than in cytoplasm. On the basis of the relative concentration of the enzyme in nucleus and cytoplasm, in fact, three types of tissue can be described. (1) Those tissues, or cell-aggregations, in which both nuclei and cytoplasm stain intensely: most tissues show this condition during the first two or three days of development, or in some cases much longer, while in several tissues this condition is achieved in the course of differentiation. (2) Those tissues in which the nuclei are more reactive than the cytoplasm: this distribution is found in some regions very early, before the peak of phosphatase concentration is reached, and again it is found commonly later, when many tissues gradually lose their early reactivity as differentiation proceeds. A decrease in enzyme content always affects the cytoplasm before the nuclei. (3) Those tissues in which neither cytoplasm nor nuclei show marked phosphatase activity: in a few cases this condition obtains throughout the period studied, but more often the relatively phosphatase-free state results from the gradual disappearance of the enzyme from originally reactive cell groups during the course of differentiation. The nuclei are never found to be less reactive than the cytoplasm, nor do they ever become completely negative, although the cytoplasm frequently does so. In the least reactive nuclei, the nucleoli stain heavily and the nuclear membranes lightly. Where the phosphatase concentration is greater, the membranes stain more darkly, the karyoplasm takes on a deepening greyish coloration, and intensely stained masses make their appearance within the membrane; these masses are probably chromosomal detritus, since the chromosomes would be expected to disintegrate during several hours exposure to a solution at pH 9.3.[2] Finally, when the phosphatase concentration is very high, the nuclei stain solid black within a relatively short time. The usual high reactivity of the nuclei makes it sometimes difficult to decide whether a change in phosphatase concentration is real, or merely the apparent result of either lessened crowding of the nuclei or an increase in the amount of cytoplasm per nucleus. To rule out errors due to these factors, it was necessary to make careful comparisons of treated sections with others stained with ordinary nuclear stains.

In the descriptions which follow, the terms "positive" and "active" or "reactive" have been applied to tissues which show more than the minimal nuclear response described above (i.e., no deep stain except in the nucleoli). Those tissues which show only the minimal nuclear response are denoted "negative." An attempt has been made to rate both alkaline and acid phosphatase concentrations on a single scale on which 16 + represents a solid black deposit appearing after 1½ hours' incubation, and 1 + the lightest recognizable deposit appearing after 16 hours' incubation.

The first day

In the unincubated blastoderm both the incipient germ layers and their neighboring yolk cells contain alkaline phosphatase. The smaller, compact yolk granules (white yolk) clustered in the marginal zone are very strongly positive (12 +), the

[2] I am indebted to Dr. Jack Schultz for pointing this out to me.

larger granulated elements (yellow yolk) being much less so. The endoderm and ectoderm are about equally positive (9 +), but their reactivity is much greater in the heavily staining nuclei and in the enclosed yolk granules than in the cytoplasm. At the periphery of the blastodermal area the phosphatase content of the ectoderm wanes.

From the blastoderm sections it appeared that the white yolk is more rich in phosphatase than the yellow, which however seemed to contain a moderate amount of the enzyme also. But smears of yolk taken from regions away from the blastoderm revealed that neither type of yolk contains much phosphatase, although both take on a spurious stain due to the presence of preformed phosphates. The high activity of the white yolk around the embryonic area thus seems rather to be related to proximity to the embryonic tissue, than to be an intrinsic property of white yolk.

As the embryonic body itself is laid down toward the close of the first day, it has at the anterior end a phosphatase content as high as in the preceding tissues, or slightly higher in the case of the ectoderm; but there is a gradual decrease in all layers toward the posterior end. Thus a fairly uniform antero-posterior gradient in intensity of staining can be observed. As before, the reaction also decreases in the superficial ectoderm of the extra-embryonic area.

Two and three days

The brain, and the mesoderm at anterior levels, stain virtually solid black during the second day (Fig. 2). Posterior to the brain the ectoderm and mesoderm are slightly less reactive, and in the entoderm the nuclei stain only moderately, while the cytoplasm is almost negative. At the posterior tip of the body all tissues are still only slightly reactive (Fig. 3). Through the bulk of the body, however, the brain, nerve cord, mesoderm, endothelia, and also the notochord, contain considerable amounts of phosphatase (8 + to 12 +). The red blood cells are negative. The nuclei of the extra-embryonic membranes occasionally show more than

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PLATE I

1, 6—acid phosphatase
2–5, 7, 8—alkaline phosphatase

1. Through primitive groove. *ec*, ectoderm; *m*, mesoderm; *en*, endoderm; *v*, vitelline membrane.

2, 3. Anterior and posterior levels of a single 44-hour embryo; note greater concentration of phosphatase in ectoderm and mesoderm at anterior level. *h*, heart; *s*, somite; *lm*, lateral mesoderm.

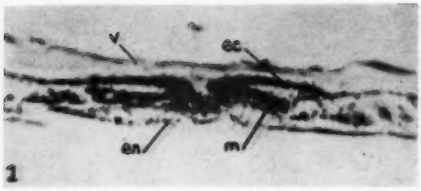
4. Through diencephalon of a 72-hour embryo; note greater phosphatase concentration in more ventral part of diencephalon.

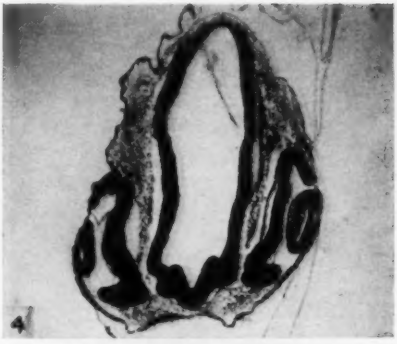
5. Through bulbus of a 5-day embryo; note high phosphatase content of valve and spongy lining tissue of bulbus. *m*, myocardium; *ct*, connective tissue; *a*, *a*, auricles.

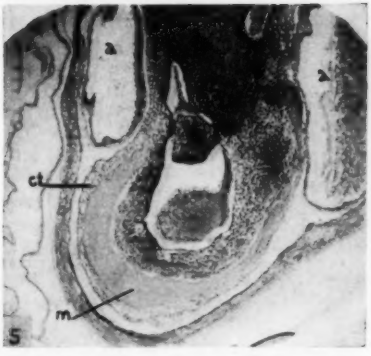
6. Through lung buds of a 5-day embryo. *a*, aorta; *b*, mesobronchus in lung bud; *o*, oesophagus; *l*, liver; *wd*, Wolffian duct.

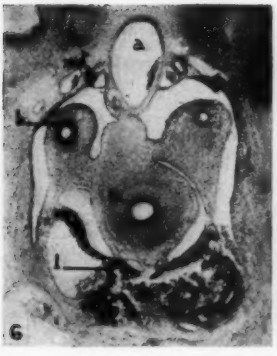
7. Differentiation of axial cartilage in fore-limb region of a 5-day embryo. *m*, phosphatase-containing membranous anlage of neural arch; *pc*, phosphatase-free protochondrium of future vertebral centrum; *n*, notochord; *a*, aorta. Note also phosphatase-free motor horns of spinal cord.

8. Liver diverticulum of 3-day embryo. *mg*, mid-gut; *l*, strongly positive liver diverticulum.









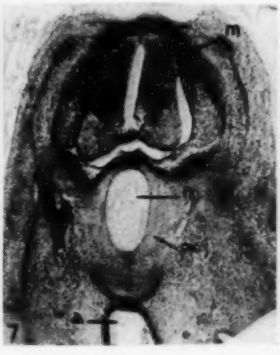


PLATE I

a minimal reaction, but more often they are negative so that there is usually a sharp line of demarcation between the embryonic and extra-embryonic tissues.

At 72 hours phosphatase is still distributed widely through the embryo, which continues to present a darkened appearance; but numerous tissues exhibit a lowered reactivity, evinced principally in the cytoplasm. This is especially true of the notochord at anterior levels, and the muscular wall of the heart.

The brain gives a strong phosphatase reaction in the most dorsal parts of the metencephalon and myelencephalon, but at about the level of the auditory capsule the hind brain is definitely less reactive (7 +) than the mid-brain, which is heavily blackened throughout. The infundibulum is moderately positive. The region of the diencephalon between the eyes appears much darker than the more dorsal region (Fig. 4), and this dorso-ventral gradation continues into the telencephalon. The dorsal part of the nerve cord and the neural crests react more strongly than the ventral part of the cord, but this difference disappears at more posterior levels, usually around the point where the amniotic folds are still open. The cranial ganglia show considerable deposits (10 +).

The eyes, the nasal placodes, and the outer faces of the auditory capsules are all strongly reactive (9 + to 10 +). The inner face of the capsule, and the lens, react weakly.

The muscular walls of the ventricle and atrium are negative, but the endothelial linings are positive. The bulbus gives a moderately strong reaction, while the walls of the ducti Cuvieri and the ventral aorta are blackened (9 +). This reactivity tends to persist in the aortic arches and dorsal aorta, but it fades in the latter as its walls become thinner toward the posterior end of the body. The cardinal veins show no particular reaction. The omphalomesenteric veins, where they enter the body, are lined with heavily reactive mesoderm, but this reactivity disappears as the vessels spread out over the yolk.

The pharynx wall is moderately positive, as is Rathke's pouch. The closely packed mesoderm in the visceral arches reacts to a lesser extent than the mesoderm of any other region. The thyroid is strongly positive. The laryngo-tracheal groove reacts noticeably, but the remainder of the alimentary canal still exhibits the low activity of the endoderm from which it is derived. The liver diverticula however are more positive than the tissue from which they arise (Fig. 8).

The mesoderm and loose mesenchyme are positive throughout the body, the re-

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PLATE II

9–15—aikaline phosphatase

9. Inner ear of 8-day embryo; note alternate light and dark staining patches of labyrinth wall. *e*, anterior semi-circular canal; *au*, acoustic ganglion; *my*, myelencephalon.

10. Optic chiasma of 8-day embryo. *oc*, central part of chiasma, just below optic nerves; *hy*, hypophysis.

11. Longitudinal section through femur of 7-day embryo, showing thick coat of perichondral phosphatase in diaphysial region. (The dark band across the epiphysis is an artefact.)

12. Transverse section through tibia and fibula of 8-day embryo. Note spongy nature of perichondral phosphatase layer, and also positive cells within the cartilage.

13. Control for 12 (opposite limb of same embryo). The stained material in the perichondral area is bone (calcium phosphate) deposited *in vivo*.

14, 15. As 12 and 13, for a 10-day embryo; section is near distal end of fibula. *e*, area of erosion.

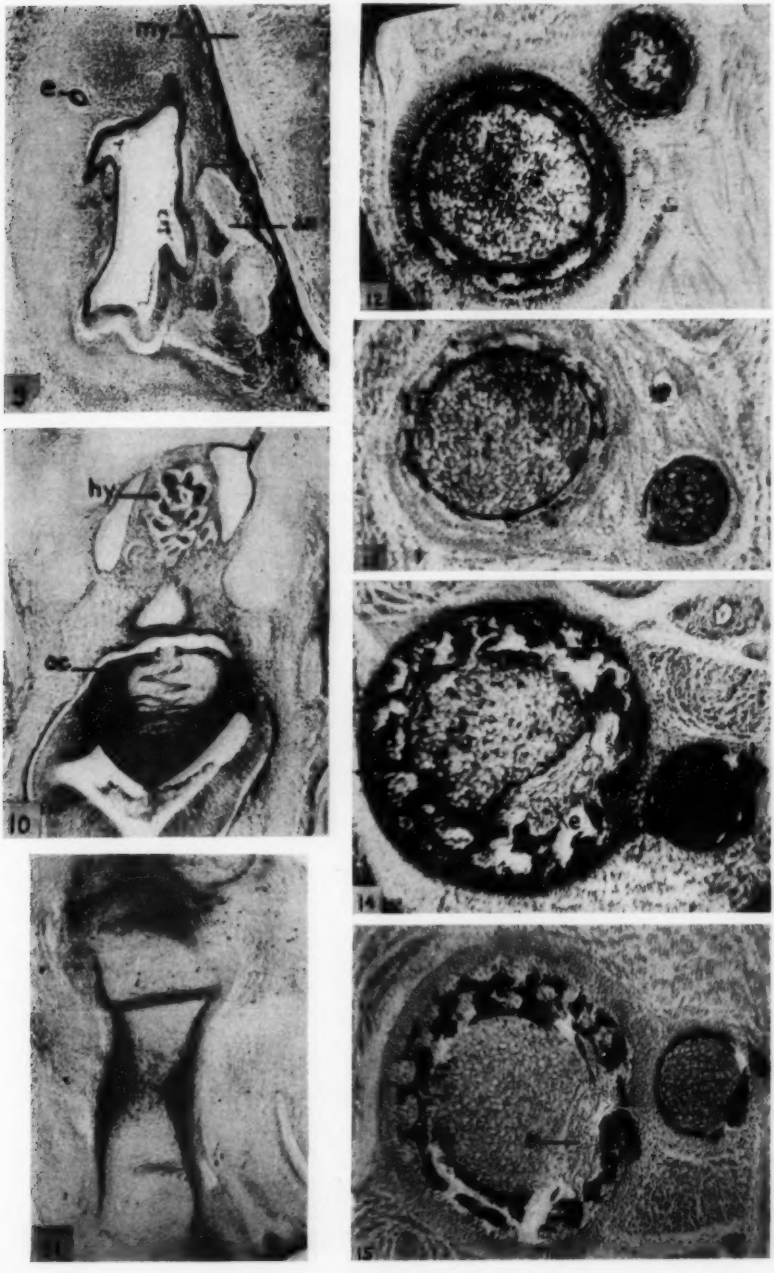

PLATE II

action still diminishing, however, toward the posterior end, as it does also in the neural tube. The nephric structures are darkened to the same extent as the surrounding mesenchyme.

Four to eight days

a. Nervous system

1. *The brain:*

Telencephalon.—The dorsal-ventral difference mentioned before is still evident in the cerebral hemispheres through the fifth day. On the ventral side there is a strongly positive region where the constriction between the hemispheres begins, but this disappears sharply where the partition is complete. Proceeding anteriorly one finds the outer and ventral faces on the hemispheres moderately positive (7 +). while the inner-dorsal faces are weakly so (4 +). The reaction gradually fades, so that the walls are almost negative near the olfactory part. On the sixth day the reactivity of the superior walls strengthens, and the phosphatase activity extends through the olfactory region.

On the seventh and eighth days the corpora striata and the pallium are lightly positive. A more intense reaction (7 +) appearing in the telencephalon medium however persists, somewhat attenuated, in the floor region of the hemispheres. The inner faces are virtually negative, as is the choroid plexus. The parencephalon is negative.

The epiphysis is negative when first formed, but phosphatase accumulates in the tubule cells as the glandular structure differentiates.

Diencephalon.—On the fourth day and later the ventral section of the posterior part of the diencephalon shows a strong reaction; but this diminishes just behind the eyes, so that for some distance between the eyes, as one proceeds anteriorly, only the central portion is strongly positive. The heavy stain at the entrance of the optic nerves, established earlier, persists; the nerves themselves are at first extremely reactive but their enzyme distribution becomes less uniform, for the differentiated supporting glia and connective tissue are negative, and only the nerve fibers retain the enzyme. As the thick walls of the thalamus develop, they show a reaction graded from moderate (5 +) dorsally to strong (9 +) on the floor. This

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PLATE III

19, 21—acid phosphatase
16–18, 20, 22, 23—alkaline phosphatase

16. Through neck of 8-day embryo. *t*, thyroid; *o*, oesophagus; *gn*, ganglion nodosum; *cc*, common carotid artery; *j*, jugular vein.

17. Part of myelencephalon of 7-day embryo. Note phosphatase-containing tissue around lumen, and sharp decrease in phosphatase content of axones from ganglion (*g*) as they enter myelenecephalon.

18, 19. Proventriculus of 8-day embryo. Note phosphatase content of mesenchyme surrounding glands. *g*, gland; *cm*, circular muscle; *ca*, coeliac artery; *l*, liver. Note that with alkaline phosphatase liver cells are unstained and endothelium stained; with acid phosphatase cells are stained and endothelium unstained.

20, 21. Mesonephroi and associated structures of 8-day embryos. *gl*, glomerulus; *st*, secretory tubule; *ct*, collecting tubule; *md*, Müllerian duct; *wd*, Wolffian duct; *go*, gonad; *mt*, metanephric tubules and blastema; *r*, rib.

22. Blood vessel and positive endothelium in liver of 8-day embryo.

23. Positive endothelium in ventricle of 8-day embryo. *rc*, red cells.

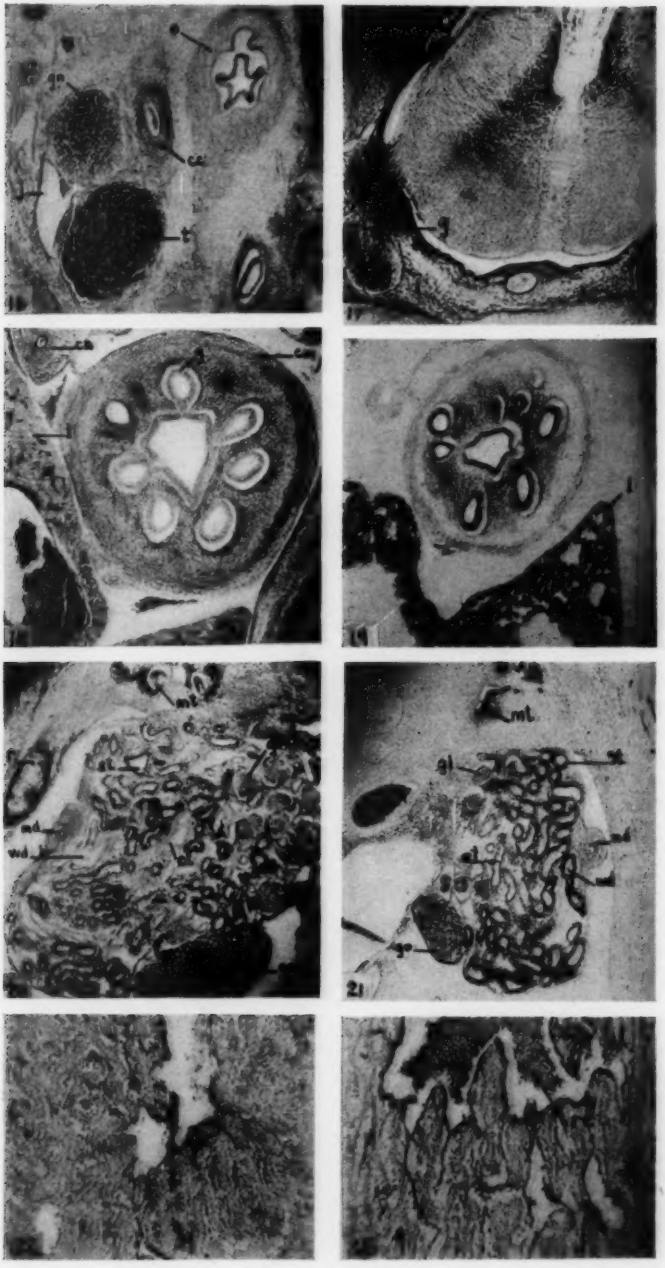

PLATE III

intense reaction is continued around the lamina terminalis, and on the eighth day the optic chiasma (Fig. 10) shows extremely heavy deposits (12 +).

Mesencephalon.—A heavy deposit (8 +) in the outer wall of the more dorsal part of the optic lobes gives the slightly reactive inner tissue the appearance of being surrounded by a shell; with the persistence of this difference the outer region comes to include the layer of short radially arranged fibers that develop near the surface on the sixth day. The ventral and posterior walls of the aqueduct are markedly positive (7 +), the dorsal and anterior walls less so (4 +). This dorso-ventral gradient, as described above, continues forward into the diencephalon.

Metencephalon and myelencephalon.—At its junction with the mesencephalon, the metencephalon shares the high phosphatase concentration of the mesencephalic floor. On the fourth day this persists for a short distance posteriorly and then fades into a more moderate reaction which grows lighter on successive days. On the sixth day, however, heavy deposits (10 +) concentrate around the outer ventral corners of the fourth ventricle and taper off into the ventro-lateral tissue. This distribution continues backward, heavy deposits remaining close to the narrowing lumen while lighter deposits (7 +) gradually spread through the whole ventral region, except for the negative raphe (Fig. 17); as the spinal cord is entered, this pattern resolves itself into the cross-band of phosphatase-rich cells which has already been described (Moog, 1943a). The thin roof of the hind brain, and also the cerebellum, are negative.

2. *The cranial ganglia:*

Throughout the period under consideration all the cranial ganglia but the eighth show marked phosphatase activity (Fig. 16) of fairly uniform intensity (10 +). The axones connecting the ganglia to the brain are positive outside the brain and negative, or weakly positive, inside, the line of demarcation being extremely sharp (Fig. 17); the processes distal to the ganglia, however, are generally much less positive. Limited regions of the auditory ganglion stain darkly, but the bulk of the ganglion, including all those portions which amalgamate with auditory structures, are negative.

3. *The spinal cord:*

The spinal cord has already been reported on (Moog, 1943a). In terms of the scale used in this paper, the highest concentrations of alkaline phosphatase in the cord may be rated as 9 + to 11 +.

4. *The sense organs:*

The ear.—On the fourth day the auditory capsule, as before, exhibits alternate patches of lightly and darkly staining tissue; as development proceeds it becomes apparent that the lighter patches are the rudiments of the maculae and cristae (Fig. 9). These areas, like the branches of the auditory nerve that fuse with them, grow less reactive and on the eighth day are virtually negative. The rest of the labyrinth wall is strongly positive (10 +), as is the entire internal limiting membrane.

The saccus endolymphaticus is variably positive, though its glands stain very deeply (11 +). The walls of the semi-circular canals stain only lightly, but the inner lining is strongly positive.

The rudiments of the Eustachian tube and of the tympanic membrane and cavity at first share the phosphatase content of the tissues from which they form, but by the eighth day they become negative.

The eye.—The eye tissue on the fourth day has generally the darkly stained appearance characteristic of most of the brain at this time, except that the median face of the cup is somewhat lighter than the rest. The nerve tract, which inserts into this less positive region of the retina, is intensely positive (10 +). The fibers of the lens core are negative, but their nuclei take a dark stain, as does the epithelial rim. Up to the eighth day the only change in the lens is that the cytoplasm of the epithelial rim loses its reactivity; the nuclei of both core and rim remain at least slightly positive throughout.

In the retina the diminution of phosphatase spreads toward the outer face of the cup on the fifth and sixth days, so that by the eighth day only the thin lenticular zone, the future iris, is markedly active. The bulk of the retina at this time is lightly positive, but the inner layer of optic fibers shows the same high phosphatase content as in the optic tract itself; and in addition there is a darkly staining layer between the ganglion cells and the base of the layer of Müllerian fibers. This reactive layer appears to include the inner plexiform fibers and also some very large cell bodies which may be the amacrine cells. In the formation of this layer both the histological differentiation and the corresponding phosphatase development spread simultaneously from the fovea outward. The mesenchymatous keel of the pecten is moderately reactive, and the budding eyelids are noticeably darkened.

The nose.—The whole nasal epithelium shares the high phosphatase content of its immediate surroundings on the fourth day, but this gradually diminishes, although the internal limiting membrane continues to stain deeply; the surrounding mesenchyme meanwhile also becomes less reactive (5 +). The condensed mesenchyme from which the nasal processes and turbinals are derived exhibits black deposits (10 +) on the seventh day, just before the cartilage begins to appear, and the nasal septum also shows phosphatase activity before the cartilaginous bar forms. As the cartilage differentiates the phosphatase again disappears.

b. Digestive system

On the fourth day the condensed mesenchyme of the visceral pouch region has a uniformly positive reaction. The lining of the mouth and pharynx stain darkly at this time, but later this epithelium loses some of its phosphatase content so that by the seventh day only the anterior and dorsal regions of the mouth lining are positive. The muscular mass of the tongue reacts moderately at the base at first, but the more anterior portion as well as the surface is only slightly positive. By the eighth day only the epithelium of the front component shows any reaction. The epithelial cords of the thyroid stain heavily throughout (Fig. 16), and the reaction is intrinsic rather than due, as one might suspect from Gomori's (1941a) results on adult mammalian thyroids, to the presence of preformed phosphate in the colloid.

The alimentary tract itself is at first moderately positive throughout, but its positivity declines steadily, with the diminution of phosphatase affecting the more posterior parts of the tract first. The reactivity of the oesophagus wanes after the period of occlusion, although it has a thin lining membrane that continues to stain heavily (Fig. 16). Between the fifth and eighth days a decreasingly positive re-

action extends through the duodenum, the lower parts of the intestine meanwhile becoming completely negative in the cytoplasm. The glands of the proventriculus include some of the positive lining membrane when first seen on the sixth day, but on the following day are quite negative (Fig. 18). On the eighth day the cytoplasm of the entire alimentary epithelium is negative, and even the nuclei, which

TABLE I

Relative phosphatase activities of digestive tract and derivatives

(For explanation of values, see page 53)

The endoderm generally has an alkaline phosphatase rating of 5+ at two to three days, acid phosphatase 1+. Mesoderm: alkaline phosphatase 7+, acid phosphatase 2+.

Tissue	Alkaline			Acid		
	4 days	6 days	8 days	4 days	6 days	8 days
Oesophagus—epith.	7+	5+	3+	1+	2+	2+
mucosa	8+	5+	4+	1+	1+	1+
Crop —epith.		4+	3+		2+	2+
mucosa		5+	3+		1+	2+
Stomach —epith.	5+			2+		
mucosa	8+			3+		
Proventriculus—epi.		4+	3+		3+	3+ (body) 5+ (glands)
muc.		7+	7+		1+	4+
Gizzard—epith.		5+	4+		2+	4+
mucosa		7+	4+		2+	2+
Small intestine—epi.	3+	2+	2+	2+	2+	4+
muc.	8+	7+	4+	2+	1+	1+
Large intestine—epi.	4+	2+	1+	2+	2+	2+
muc.	8+	7+	4+	1+	1+	trace
Rectum	3+	2+	2+	1+	1+	trace
Cloaca	1+	1+	1+	trace	trace	trace
Pancreas	4+	4+	3+*	2+	3+	3+
Liver cells	7+	5+	2+	4+	5+	6+
Liver endothelium	9+	10+	12+	trace	trace	trace
Ductus choledochus		3 to 8+	3 to 6+	2+	3+	4+
Thyroid	10+	12+	12+	3+	5+	7+
Trachea	8+	5+	4+	2+	3+	4+
Bronchi	8+	5+	4+	2+	3+	4+
Mesobronchi	8 to 3+	7 to 3+	2+	2+	3+	4+
Entobronchi		3+	3+		2+	2+
Mesoderm of lung bud	8+	7+	4+	2+	1+	1+

* In tubules, about 8+.

were originally strongly positive, retain only a small part of their former reactivity. The phosphatase deposits also disappear from the linings of the crop and proventriculus, but those of the gizzard and duodenum remain positive. The cloaca is negative throughout.

The closely packed mesenchyme surrounding the developing alimentary tract gives a moderately strong reaction at first, and this continues to be true on the fifth

and sixth days, as the mesenchyme becomes differentiated into a layer of loosely packed tissue (mucosa) immediately around the digestive tube, and a denser layer of circularly arranged cells (muscularis mucosa) outside the first. By the eighth day, however, the muscular layer has lost most of its phosphatase, except for small amounts in the nuclei, but the inner layer continues to be reactive (Fig. 18).

The pancreatic tissue, as it differentiates, becomes less reactive than the mesoderm in which it lies. On the sixth or seventh day, however, phosphatase begins to appear in the organ itself, and on the eighth day, when a glandular structure begins to develop, the tubules are strongly positive. The liver has a strong reaction at four days, but with the progress of differentiation the center of the mass of tissue first becomes negative, and this negativity gradually extends to the periphery; by the eighth day even the nuclei of the hepatic cells have only a trace of activity. During the same period, however, phosphatase develops in the endothelium lining the sinusoids, the reaction appearing first around the principal venous channels and then spreading through all the liver spaces (Fig. 22). The ductus choledochus is reactive within the liver, but becomes negative as soon as it emerges from the liver substance.

The trachea is strongly positive on the fourth day, but the reactivity wanes steadily. The more proximal portions of the bronchi react similarly, but it can be seen on the fifth day that the phosphatase content diminishes sharply at the bend between the anterior and middle mesobronchi, and again between the middle and posterior mesobronchi. Both trachea and mesobronchi have an extremely black inner lining at first, but this too gradually loses its enzyme content. The entobronchi are almost negative throughout.

If the trachea of the hatched chicken has the same high concentration of phosphatase in its ciliary border as Bourne (1943) has described for the trachea of the rat and guinea pig, the accumulation of phosphatase must occur after the eighth day.

c. Urogenital system

Although it differentiates from active mesenchyme, the cytoplasm of the Wolffian body is generally weakly positive on the fourth day. The only parts which show an intense reaction are the necks of the tubules, and the nuclei throughout. The cytoplasm of the duct itself is clear along its entire length. As the structure differentiates further, a high concentration of phosphatase accumulates in the brush borders of the secretory tubules, while the quantity of the enzyme diminishes simultaneously in the outer borders of these tubule cells. The collecting tubules, Bowman's capsules, and the glomeruli, as well as the Wolffian duct, have little or no phosphatase during this period. The Müllerian duct however stains quite darkly (Fig. 20). The metanephric blastema is moderately positive, but the tubules that differentiate from it are only weakly so; the blastema of the ureter, on the other hand, contains only a little phosphatase, and the tube itself shows no change in this respect.

The gonads are quite reactive when they first appear, but their phosphatase content diminishes steadily. The same is true of the suprarenals. Neither organ had assumed a pattern of phosphatase distribution, as the findings of Gomori (1941a) and Kabat and Furth (1941) suggest that they might at a later stage.

TABLE II

Relative phosphatase activities of the urogenital system

At 2 to 3 days: mesoderm from which Wolffian body is formed has alkaline phosphatase rating of 8+; acid phosphatase, 2+.

Tissue	Alkaline			Acid		
	4 days	6 days	8 days	4 days	6 days	8 days
Glomeruli	3+	3+	3+	1+	1+	1+
Bowman's capsules	5+	3+	3+	trace	trace	trace
Secretory tubules—walls	10+	6+	3+	4+	4+	4+
—brush border	10+	14+	14+	4+	4+	4+
Collecting tubules	4+	trace	trace	2+	2; 8+	2; 8+*
Wolffian duct	4+	3+	2+	8+	8+	8+
Müllerian duct		8+	5+		2+	2+
Metanephric blastema		8+	8+	2+	2+	2+
Metanephric tubules		4+	4+	8+	8+	8+
Ureter	4+	4+	3+	2+	2+	2+
Suprarenal (provisional cortex)	8+	6+	4+	1+	1+	2+
Gonad	10+	8+	6+	2+	3+	4+

* The phosphatase content of the tubule rises sharply at its entrance into the Wolffian duct.

d. Circulatory system

The heart.—The cardiac muscle continues to be completely free of alkaline phosphatase; even the nuclei, which stain lightly on the fourth day, become completely negative by the eighth day, except that the nucleoli are still reactive. Thus a clear-cut boundary appears in the bulbus between the negative heart tissue and the phosphatase-containing tissue external to it. The thin endothelial linings, which by the fifth day reach into the deepest recesses of the trabeculae, stain solid black (Fig. 23). High concentrations of phosphatase are also found in the septa, including the cushion septum, a development which is already foreshadowed on the third day by thickening of the intensely reactive epithelium around the valves. The thick spongy lining of the bulbus shares this positive reaction (Fig. 5); in fact, all the vessels entering or leaving the heart are lined with phosphatase containing tissue.

The blood vessels.—When first laid down, all the blood vessels share the high reactivity of the condensed mesenchyme that forms them (Fig. 7). As late as the eighth day the still poorly differentiated small arteries are reactive throughout, but the walls of the aorta and the larger arteries gradually become free of phosphatase as the muscular tissue differentiates. However the pulmonary arteries, and the aorta and its major branches (Fig. 18), are lined with highly reactive endothelium. The umbilical artery has virtually no phosphatase by the eighth day, while the omphalomesenteric arteries have a reactive endothelium where they branch from the aorta, but this becomes negative as they pass outside the body.

The veins are not generally reactive (Fig. 16). The ducts of Cuvier and the sinus venosus, however, and later the proximal portions of the venae cavae, possess

the same phosphatase-rich lining as the heart itself. Likewise the meatus venosus and hepatic veins are lined with the heavily staining tissue that runs through the sinusoids, and the omphalomesenteric veins remain moderately positive throughout the period studied. The larger umbilical vein is rather strongly positive. Except for the capillary bed of the liver, the walls of the capillaries are by the eighth day not more reactive than the tissues that form them.

The presence of alkaline phosphatase in vascular endothelia at the early stages at which it has been observed is of interest in relation to the question of the origin of endothelial phosphatase. It is sometimes held that endothelial cells pick up phosphatase from the serum. If Armstrong and Banting (1935) are correct, however, in their contention that bone is the source of serum phosphatase, it is clear that phosphatase in the endothelium of six- or seven-day embryos cannot be derived from serum; more likely the endothelia, like other tissues, produce their own phosphatase.

The spleen has a weak, uniform reaction through the eighth day, at which time it is still in an early state of differentiation.

e. The skeleton

The hind limbs from the sixth to the tenth day were studied in most detail; pairs of limbs were fixed on each day, plus pairs of feet for the eleventh day, one member of each pair being used for phosphatase demonstration and the other for the demonstration of phosphate deposited *in situ*. All specimens were sectioned transversely.

The limb buds on the fourth day are merely masses of condensed mesoderm which share the high phosphatase content common to mesoderm at this time (8 to 10 +). On the fifth day each mass becomes differentiated into an inner portion, the rudiment of the cartilage, which is more condensed and highly reactive; and an outer portion, destined to form muscle and connective tissue, which is comparatively rarified and less reactive. As the precartilages become transformed into protochondrium, their phosphatase content diminishes sharply except at the periphery, where a thin layer of compressed cells remains positive (8 +); within the mass the nuclei continue to show a fairly strong reaction, but the cytoplasm and developing matrix become quite negative. During the protochondrial stage the enveloping layer of phosphatase-containing cells increases in thickness without giving a more positive reaction; but with the appearance of true hypertrophic cartilage the perichondrium begins to develop the most intense reaction found in the early embryo (16 +); the cells affected are the perichondrial osteoblasts.

As is true of histological differentiation, the accumulation of phosphatase begins, in the long bones, midway along the diaphysis, and spreads gradually toward the epiphyses; simultaneously the layer increases in thickness, becomes open and spongy (Figs. 12 and 14), and its inner edge invades the matrix raggedly. In the fibula phosphatase appears throughout the diameter of the shaft toward the distal end, but where typical epiphyses occur the phosphatase accumulation stops abruptly at the zone of flattened cells (Fig. 11). When the phosphatase-laden osteoid has achieved a thickness in the diaphysis roughly equal to half the radius of the enclosed cartilage, thin shells of bone are laid down near the inner surface of the layer (Figs. 13 and 15). From Table III, which summarizes the development of carti-

lage, phosphatase, and bone in the hind limb from the sixth to the eleventh day, it will be seen that bone deposition follows inevitably when a certain level of cartilage differentiation is reached; this is equally true for the tibia, in which the development of the cartilage and osteoid envelope is rapid, and for the proximal phalanges, in which hypertrophy and phosphatase accumulation are slow.

It was pointed out above that the first differentiation of cartilage from mesoderm is accompanied by a marked diminution of the primitive embryonic phosphatase in

TABLE III

Summary of cartilage, alkaline phosphatase, and bone formation

Day	Structure*	Condition of cartilage	Condition of perichondral phosphatase layer	Deposition of perichondral bone
6	tibia, fibula	precartilage	light (8+)	−
	femur	precartilage	light (8+)	−
	metatarsals	mesenchyme	—	−
7	phalanges	precartilage	light (8+)	−
	metatarsal 1	mesenchyme	—	−
	metatarsal 2–4	protochondrium	2-thin, compact (12+)	−
			3, 4-thick, compact (16+)	−
	tibia, fibula	hypertrophic	thick, compact (16+)	−
	femur	hypertrophic	thick, compact (16+)	−
8	phalanx 1	precartilage	light (8+)	−
	phalanx 2–4	protochondrium	thin, compact (12+)	−
	metatarsal 1	precartilage	light (8+)	−
	metatarsal 2–4	hypertrophic	thick, spongy (16+)	3, 4+; 2−
	tibia, fibula	hypertrophic	thick, spongy (16+)	+
	femur	hypertrophic	thick, spongy (16+)	+
9	phalanges	protochondrium	thin, compact (12+)	−
	metatarsal 1	protochondrium	thin, compact (12+)	−
	metatarsal 2–4	hypertrophic	thick, spongy (16+)	+
	tibia, fibula	hypertrophic	thick, spongy (16+)	+
	femur	hypertrophic	thick, spongy (16+)	+
10	phalanges	hypertrophic	thick, spongy (16+)	−
	metatarsal 1	protochondrium	thin, compact (12+)	−
11	phalanges	hypertrophic	thick, spongy (16+)	+

* The most advanced portion of the structure is considered.

the cytoplasm. Shortly afterward the nuclei in the zone where hypertrophy occurs also undergo loss of phosphatase, so that the cells of the cartilage beginning to hypertrophy are negative. Already on the seventh day in the tibia and femur, however, some of these cells develop a very intense reaction while other neighboring cells continue to be negative; at the same time the matrix begins to exhibit signs of phosphatase activity. The reactive cells seem to decrease in number through the tenth day, and they are most numerous midway along the diaphysis. Possibly these cells are engaged in synthesizing the phosphatase that becomes con-

centrated at the periphery, or they may be endochondral osteoblasts, for erosion has begun in advanced cartilages on the tenth day (Fig. 14).

The anlagen of the vertebral bodies and neural arches pass through the same stages of cartilage differentiation as do those of the long bones, at about the same time, although by the eighth day the hyaline cartilage of the axial skeleton has achieved an advanced state of differentiation only immediately around the notochord (Fig. 7). Each separate element is enveloped by a compressed layer of elongate cells which are more reactive than those within the cartilage; this layer resembles that which envelops the small-celled cartilage of the long bones, but it never has anything like the tremendous phosphatase activity of the perichondrium of ossifying long bones. The cell bodies are generally only slightly reactive, but in the anterior region on the eighth day a number of reactive cells appear among the negative ones in the center of each future vertebra, where endochondral ossification will begin several days later. In a few sections through the neck of an eleven-day embryo it was observed that both matrix and cells of the cartilage close to the notochord contained a very high concentration of phosphatase, while the remainder of the vertebral cartilage contained none of the enzyme; ossification had not begun.

No detailed study was made of the development of the girdles, but it was noted that on the eighth day the coraco-scapula is covered by a phosphatase-rich perichondrium (14 +) similar to that of the limb bones; the layer is thicker on the coracoid than on the scapular part. The clavicle at the same time is a mass of mesoderm heavily impregnated with phosphatase. The elements of the pelvic girdle show practically no perichondral phosphatase accumulation at eight days. The ribs however are heavily coated with strongly reactive phosphatase in the diaphyseal regions (Fig. 20).

The development of the skull was not studied.

f. Other tissues

Notochord.—The nuclei of the notochord are less intensely reactive on the fourth day than at earlier stages, and the cytoplasm is negative. Within the next two days the nuclei also lose the remainder of their phosphatase content.

Muscles.—As the fibers of the skeletal muscle differentiate, their cytoplasm loses entirely the phosphatase content of the primitive mesoderm from which they are formed. The nuclei lose most of their phosphatase, but even on the tenth day they are still slightly reactive.

Skin.—The superficial ectoderm is intensely reactive at the beginning of embryogenesis, but already by the fourth day the phosphatase content of the cytoplasm has diminished sharply, and by the seventh day even the nuclei are negative. At the same time the thick mesodermal corium has also become negative. This change is not uniform, but begins first in the dorsal and anterior regions of the body.

Mesenchyme.—The loose mesenchyme distributed throughout the embryo is positive (8 +) as late as the fifth day, but thereafter it gradually loses its enzyme content. On the eighth day this tissue is almost negative in the body, but in the head masses of it which are presumably concerned in the ossification of membrane bone remain strongly positive.

Feather germs.—Undifferentiated feather germs were found on ten-day hind limbs. They contained a core of positive mesoderm (8 +).

II. Acid Phosphatase

Acid phosphatase is widespread in the early embryo, but in virtually all cases in apparently far lower concentration than alkaline phosphatase. With Gomori's (1941b) unmodified technique only the Wolffian duct and some of the kidney tubules ever produced a black deposit; in all other organs the deposits, even after 16 hours' incubation, took merely a golden-brown stain of varying intensity. As has been noted before, the extent and intensity of staining varied considerably among different specimens of the same age, and in spite of the preparation of a large number of specimens it was not easy to decide how much of the variability is intrinsic and how much due to the troublesome lability of the enzyme. The use of ascorbic acid as an activator improved the uniformity of the results but, as will be explained later, it did not entirely correct the inconstancies. Several embryos even failed to produce any deposits at all, for no readily apparent reason. When deposits did appear, however, their distribution and relative concentrations in the separate organs and throughout the body as a whole were quite constant.

Acid phosphatase is commonly found in nuclei, but unlike the alkaline enzyme it occurs only in uniform distribution in the karyoplasm, which takes a diffuse stain; generally in reactive cells, however, there is a darkly stained cap, which may be the Golgi apparatus, closely applied to the surface of the nucleus (cf. Moog, 1943). Bourne (1943) found such caps stained for alkaline phosphatase in the nuclei of certain intestinal cells; they were not observed in the alkaline-stained cells of the embryo, but the heavy reactions of the nuclear sap and membranes may have obscured them. On the basis of the relative distribution of the enzyme in nucleus and cytoplasm the same three classes of tissues may be defined as were described for alkaline phosphatase; but in addition, in the differentiation of some tissues acid phosphatase disappears almost completely from the nuclei as well as from the cytoplasm.

The difference in the nuclear staining is one argument against the possibility that the acid enzyme of this study is merely residual activity at acid pH of the much stronger alkaline enzyme. And this possibility is completely eliminated by several other facts. First, the alkaline enzyme is entirely suppressed at pH 8. Second, at later stages the two enzymes assume distributions that differ in many important respects. Third, at early stages as well as later, the acid enzyme is inhibited by fluoride, which has been shown to have no effect on the alkaline enzyme. And last, as will be demonstrated in a subsequent section, the two enzymes are affected in opposite fashions by a variety of chemical substances.

The first day

In the unincubated blastoderm the smaller yolk granules, whether in the superficial layers of the yolk or enclosed within the cells, show a moderately high phosphatase content, while the deeper layers of yolk are less reactive; but as in the case of the alkaline phosphatase, proximity to the blastoderm rather than the nature of the yolk, seems to determine the enzyme content. The central area of the ectoderm is the most reactive part at this time (about 3 +); the reactivity of the cytoplasm wanes somewhat near the germ wall. The endoderm has little cytoplasmic phosphatase, although the yolk granules enclosed in the cells cause it to stain no-

ticeably. The nuclei are difficult to detect, and do not seem to be stained more deeply than the cytoplasm.

As the primitive streak differentiates, the ectoderm composing it becomes more reactive (4 +), and the demarcation between the central ectoderm and the less reactive periphery becomes sharper. The endoderm stains more intensely than in the gastrula stage, and the mesoderm is intermediate in enzyme content between the ectoderm and endoderm; as the yolk granules disappear it can be seen that in the mesoderm and endoderm the nuclei are more reactive than the cytoplasm, but in the ectoderm the intensity of the cytoplasmic stain obscures the condition of the nuclei (Fig. 1). The head process ectoderm and the notochord share the high phosphatase content of the primitive streak ectoderm as soon as they are laid down, while the surrounding extra-embryonic tissue continues to be only weakly reactive (1 +). The activity of the lateral mesoderm increases when the somites begin to form.

Two and three days

The nervous system continues to be the most reactive on the second day; the notochord is also quite reactive, but the other tissues of the body are markedly weaker (2 +), although their nuclei stain rather deeply. The extra-embryonic tissues become negative at a short but variable distance from their confluence with the body. The same antero-posterior gradient obtains as was noted in the case of alkaline phosphatase.

On the third day the spinal cord is as rich in phosphatase as before, and in the ependyma sometimes even more so (4 +). In certain regions of the brain, however, there is a sharp decrease. The hindbrain continues to be reactive, especially where it passes into the spinal cord, but the midbrain is largely unreactive, except in its ventral region, and the diencephalon too remains positive only on its roof and around the point where the optic stalks enter. Of the telencephalic vesicles only the anterior faces react. The nasal pits, optic cups and lenses, and otocysts continue to be positive, though less so than the spinal cord.

The loose mesenchyme is negative, but condensed aggregations of mesodermal cells remain lightly positive (2 +). There is a sharp increase concurrent with the appearance of the Wolffian body, however, and already at the end of the third day the Wolffian duct is quite rich in acid phosphatase (8 +). The endoderm continues to be weak except at the points of the liver and allantoic diverticula: the liver tissue is extremely reactive (4 to 5 +) when it first appears, and the allantois is equally reactive; the mesoderm of the allantoic bud shares the strong reaction of the endoderm.

Four to eight days

a. Nervous system

1. *Brain and cranial ganglia:*

The brain continues to show some acid phosphatase activity through the fifth day. At that time the mid- and hindbrain are about equally reactive (2 +), with the ependymal layer being more positive than the rest; otherwise there are no clear-cut local differences. The bulk of the diencephalon contains only a trace of acid phosphatase, but the dorso-lateral walls, and the ventral portion where the optic

nerves enter, are slightly more reactive (1 +). The cerebral hemispheres can also be rated at 1 + to 2 +, except that the partition which separates them is negative. On the sixth day the quantity of acid phosphatase in the brain is still slighter, and on the seventh and eighth days only with the aid of ascorbic acid could traces of the enzyme be detected. Similarly the acid phosphatase content of the cranial ganglia falls to a mere trace by the eighth day.

2. *Spinal cord:*

The general phosphatase activity described for the spinal cord (Moog, 1943a) is of the order of 2 + to 3 +, with the concentrations in the motor horns and ependyma being considerably stronger (4 + to 5 +). The activity of the large cells of the ganglia can also be rated at 5 +.

3. *Sense organs:*

The sense organs in their earliest expressions share the phosphatase content of the ectoderm from which they are formed. Their reactivity wanes rapidly, however, and by the sixth day they are all devoid of phosphatase, except that traces remain in the retina.

b. Digestive system

The condensed mesenchyme of the visceral pouch region exhibits only slight activity on the fourth day, and even this small activity wanes subsequently. The epithelial lining of the mouth is almost negative, but the inner surface of the pharynx and the laryngotracheal groove retain some acid phosphatase activity throughout this period. The thyroid stains heavily, according to the same pattern as with alkaline phosphatase.

The oesophagus gains somewhat in activity on the fifth or sixth day (Fig. 6), attaining a moderate phosphatase content which it shares with the crop; the inner lining membrane does not stain more deeply than the cells. The mesenchyme surrounding the oesophagus is uniformly weak, but that around the crop by the eighth day exhibits a relatively high phosphatase content in the innermost layer of compact tissue; the intermediate layer of loose mesenchyme and the outer layer of involuntary muscle fibers are much less active. The stomach and intestinal endoderm increase markedly in enzyme content up to the eighth day. The body of the proventriculus accumulates considerable phosphatase, and the glands become even richer (Fig. 19); the outer mesenchyme shows the same reactions as just described. The endoderm of the gizzard is also increasingly active, but its mesenchyme, which is less differentiated than that further anterior, is only slightly active on the eighth day. The small intestine shows the same relations as the gizzard, but the endoderm of the large intestine and rectum continue to be only weakly positive, and their accompanying mesenchyme contains only traces of phosphatase. The cloaca develops a very small enzyme content during this period.

The liver cells continue to gain in phosphatase content as the organ grows (Fig. 19), the increase proceeding from around the large veins to the periphery; the sinusoidal linings however do not contain the acid enzyme. The ductus choledochus is positive, but less so than the liver tissue surrounding it. The pancreas is at first

only slightly positive, but as in the case of alkaline phosphatase there is some accumulation of the acid enzyme as the glandular structure begins to differentiate.

The trachea and the respiratory tubes which spring from it undergo the same small increase in phosphatase content as occurs in the digestive tract generally between the fourth and the eighth day (Fig. 6), though the entobronchi remain less positive than the rest. The mesoderm of the lung bud, on the other hand, is only faintly reactive throughout.

c. Urogenital system

The Wolffian duct continues to be the most phosphatase-rich organ in the body (Fig. 6). The tubules are at first equally reactive throughout, but by the fourth or fifth day the secretory portions of the tubules begin to show a heightened reaction both in the brush borders and throughout the cells of the tubule walls; this is the same condition as Gomori (1941b) found in the kidneys of several adult mammals. The collecting tubules are definitely less reactive except at their entrance into the Wolffian duct, where they suddenly exhibit the intensity of the duct itself. The glomeruli are slightly positive, and Bowman's capsules have merely a trace of activity. The Müllerian duct is moderately positive (Fig. 21). The metanephric blastema is only lightly positive, but the tubules display very high activity, almost equal to that of the Wolffian duct, as soon as they appear. The ureter is weakly positive.

The gonads seem to possess a uniform overall phosphatase content during the period under consideration. But by the eighth day the enzyme is concentrated principally in the cortex and sex cords, the interstitial substance being much less reactive (Fig. 21). The suprarenal increases slightly in reactivity by the eighth day, but, as is also true of the alkaline enzyme, close examination fails to reveal differentially stained elements which might be the chromaffin cells.

d. Circulatory system

With the exception of the spongy lining tissue of the bulbus, which regularly demonstrates a small phosphatase content, all parts of the circulatory system have generally been found to be negative (Fig. 6). In one case, however, in which freshly prepared specimens were incubated for 18 hours in the presence of 0.01 M ascorbic acid, traces of acid phosphatase were found in all parts of the circulatory system; there appeared to be no noteworthy local differences. The red blood corpuscles are negative.

The spleen has a low phosphatase content throughout the eighth day.

e. Other tissues

The loose mesenchyme has only a trace of acid phosphatase activity, but the condensed masses of the tissue retain their phosphatase content until differentiation occurs. The membranous anlagen of the cartilages are thus somewhat positive (2+) on the sixth day, but as the definitive cartilage appears it is negative, except for small amounts of phosphatase in the cell nuclei. The acid enzyme does not appear to have any further role in the differentiation of cartilage bones; its possible relation to the differentiation of membrane bones was not examined.

The striped muscles similarly lose practically all of their acid phosphatase content as they differentiate from reactive mesoderm.

The notochord is virtually negative by the fifth day, except for traces of phosphatase remaining in the nuclei.

The skin also loses most of its primitive reactivity by the sixth day, after which time all layers are negative, except, again, for slight reactivity in the nuclei.

Chemical Tests

The purpose of determining the effects of various chemical reagents on the activity of the embryonic phosphatases is threefold: first, to discover to what extent the embryonic phosphatases are identical with the phosphatases which have already been found in a variety of tissues and secretions; second, to find out whether each enzyme is a single entity or shows local variations; and third, to elucidate the differences between the two enzymes, and incidentally examine the possibility that any part of the weak activity of the acid enzyme is merely residual activity at acid pH of the stronger alkaline enzyme. Six-day embryos are most suitable for these purposes, since at this stage both alkaline and acid enzymes have to a certain degree assumed what appears to be their definitive distribution in partly differentiated organs, yet at the same time large amounts of both enzymes are still present in their primitive state in undifferentiated tissues. In order to secure a large number of comparable slides for treatment, a ribbon 8 micra thick was cut from the embryo and consecutive sections in groups of two or three placed on slides; in this way a series of 12 or more slides all showing practically the same tissues could be obtained. Such series were made at the eye, fore-limb, mid-body, and hind-limb level. Incubation was continued for 1½ hours in tests for the alkaline enzyme, 15 hours for the acid enzyme.

pH.—The alkaline phosphatase activity was weakened at pH 8.6 and abolished at pH 8.0. No acid phosphatase activity was obtained at pH 4.7 or pH 5.4.

Mg^{++}.—0.01 M $MgCl_2$ markedly activates the alkaline enzyme, at least doubling its production of phosphate during a one-hour run. A 0.001 M solution has a slighter effect. A 0.01 M solution does not affect the activity of acid phosphatase. These results are in agreement with the generally accepted hypothesis that Mg^{++} is a coenzyme of the alkaline phosphatase, but has no relation to the acid enzyme.

Mn^{++}.—The peculiar effect of $MnSO_4$ on the precipitate formed under the influence of acid phosphatase has already been reported (Moog, 1943b); it was concluded that no true activation is involved. The effect of $MnSO_4$ on the alkaline enzyme could not be tested, since its addition to the alkaline incubation solution causes the formation of a precipitate.

Zn^{++}.—Hove, Elvehjem and Hart (1940) reported that Zn^{++} increases the activity of crude alkaline intestinal phosphatase, decreases that of kidney and bone, and inhibits all three after dialysis. In the embryo 0.001 M $ZnSO_4$ greatly inhibits the activity of alkaline phosphatase, while 0.01 M abolishes the activity. Neither concentration had any effect on acid phosphatase.

F^-.—NaF is commonly accepted as an inhibitor of acid but not of alkaline phosphatase. 0.01 M NaF completely inhibited the acid enzyme in these studies; it could not be tested against the alkaline enzyme because of the very low solubility of CaF_2.

Sodium glycocholate.—Bodansky (1937) reported that various bile acids, including glycocholate, inhibit bone and kidney, but not intestinal, alkaline phosphatase; also Schmidt and Thannhauser (1943) found intestinal phosphatase to be unaffected by bile acids. In nine-day embryos 0.01 M Na glycocholate completely suppressed the activity of alkaline phosphatase in heart and liver endothelia, nerve and osteoid tissue, kidney and gonad, and also in the epithelium, brush border, and mucosal mesenchyme of the small intestine.

Phloridzin.—Lundsgaard (1933) originally showed that phloridzin inhibits both phosphorylation and dephosphorylation by kidney phosphatase. Later Kalckar (1936) and Beck (1942) substantiated the claim that phloridzin suppresses the phosphorylation of glucose by kidney extracts at neutral pH; but Beck also reported that phloridzin inhibits the hydrolytic activity of kidney phosphatase at pH 5 but has no effect at pH 7–9; the latter finding is in agreement with the results of numerous other workers, including Kritzler and Gutman (1941), who found that 0.01 M phloridzin has no effect on the alkaline phosphatase activity of the rat kidney in either the direct chemical or histochemical technique. The embryonic alkaline phosphatase, however, was very strongly (though not quite completely) inhibited by 0.01 M phloridzin, whereas the acid phosphatase was but slightly affected by the same concentration.

Reducing substances.—Albers (1935) presented extensive evidence to show that kidney phosphatase is inhibited by sulfhydryl compounds, and his results have several times been substantiated wholly or in part (del Regno, 1939; Pyle, Fisher and Clark, 1937; Schmidt and Thannhauser, 1943). In this study it was found that the alkaline phosphatase activity is suppressed completely by 0.01 M glutathione and by 0.01 M cysteine hydrochloride; and it is also inhibited to a marked extent (estimated as 50 per cent) by 0.01 M ascorbic acid. (Possibly rapid inactivation of ascorbic acid at pH 9.3 accounts for the incompleteness of this inhibition; however, Kiese and Hastings (1938) found that in runs lasting less than an hour 0.01 M ascorbic acid also had only a slight effect.) Acid phosphatase could not be tested with the two sulfhydryl reagents, since their addition to the acid incubating solution caused the appearance of a heavy precipitate. But 0.01 M ascorbic acid activated the acid phosphatase in freshly prepared specimens to an extent that seemed reasonable to estimate at 300 per cent.

From the effect of ascorbic acid it was first thought that the loss of acid phosphatase activity that histochemical preparations commonly undergo might be due entirely to oxidation. Therefore the effect of ascorbic acid on sections which had been standing at room temperature for one week was compared with that on freshly prepared sections. Activation occurred in the older material, but the level of activity was not as high as that of activated fresh preparations. Hence it appears that the spontaneous loss of acid phosphatase activity is not due principally to oxidation reversible by ascorbic acid.

Oxidizing agents.—The strong effect of ascorbic acid on acid phosphatase nevertheless indicated the advisability of determining the effect of oxidizing agents on the enzyme. Although Barron and Singer (1943) have reported that acid phosphatase is not inhibited by sulfhydryl oxidants, the activity of the enzyme in the presence of 0.01 M iodoacetic acid was tested: there was no clearcut inhibition. On the other hand, the activity of alkaline phosphatase was almost completely suppressed by 0.01 M iodoacetic acid.

It was then decided to try the effect of a less specific oxidizing agent. In the case of the alkaline enzyme, 0.01 M potassium ferricyanide exerted a limited but definite inhibition, estimated as 30 to 40 per cent. In the case of the acid enzyme, complete inhibition occurred. This inhibition was quite reversible, as was shown by exposing sections to incubating solution containing ferricyanide for two hours, and then transferring to plain incubating solution; the deposits produced were equal to those produced in control sections not exposed to the oxidizer. According to Sizer, both acid and alkaline phosphatase are inactivated by only such oxidizing agents as have E_h values between + 400 mv. and + 600 mv., the range in which 0.01 M ferricyanide probably falls; the fact that the E_h would be higher in acid than in alkaline solution may thus account for the more severe effect on the alkaline enzyme.

Taken together, these results with oxidizers and reducers offer little support for the view that sulfhydryl groups are important in the functioning of phosphatases. Particularly in the case of the alkaline phosphatase, that view does not seem reconcilable with inhibition by both iodoacetic acid and — SH compounds. The acid phosphatase moreover has now at least twice been shown to be insensitive to the effects of sulfhydryl oxidants.

The purposes of the chemical tests have been clearly fulfilled. In the first place, it has been shown that the two embryonic enzymes correspond well with the patterns that extensive research has delineated for alkaline and acid phosphatases generally. With one important exception, such differences as have been indicated can be explained as being due to differences in the state of purification of the enzymes; the exception is of course the action of phloridzin. Presence or absence of protective substances, coactivators, etc., probably cannot be called into account here, at least for the alkaline enzyme, for Kritzler and Gutman made histochemical tests of the latter on material prepared just as in this study, except that they used alcohol as fixative. The situation is the more difficult to understand because the kidney phosphatase has by the sixth day assumed what has been repeatedly shown to be its definitive distribution (Gomori, 1941a; Kabat and Furth, 1941; Krugelis, 1942), and furthermore may be assumed to be functional; yet the embryonic kidney phosphatases do not give the same response to phloridzin as other kidney phosphatases have been found to give.

Secondly, it has been amply demonstrated that the two embryonic phosphatases are separate substances. In addition to the fluoride sensitivity of the acid enzyme, it has been shown that the two enzymes differ, both in primitive and in differentiated tissues, in their responses to Mg, Zn, phloridzin, ascorbic acid, and iodoacetic acid.

Thirdly, no evidence has been found to indicate that each enzyme is other than a single entity. Taking into consideration the varying concentrations of the enzymes in different tissues, it may be stated that in no case was one tissue affected more or less than others by any reagent. This finding is in agreement with most current work, for no attempt to fractionate the phosphomonoesterases which Folley and Kay (1936) have classified as AI (alkaline) and AII (acid) has succeeded, except that of Bodansky, cited above, whose results may be due to factors extrinsic to the enzyme proper.

Lastly, no differences have been found between nuclei and cytoplasm in regard to the characteristics of either phosphatase. This result was partly to be expected from the fact that the nucleotidase which Levene and Dillon (1930) and others have found in numerous animal tissues is now accepted as being identical with the common alkaline phosphomonoesterase, with which Folley and Kay indeed classified it. The acid phosphatase of nuclei, on the other hand, has apparently been neglected; the evidence presented here certainly indicates that it does not differ from the cytoplasmic acid phosphatase.

Discussion

The most significant result to emerge from this study may be the fact that all primitive embryonic tissues, and especially all nuclei at early stages, contain phosphatase, both alkaline and acid. That phosphatase may occur in tissues which are far from assuming their definitive function has been demonstrated before by Kabat and Furth (1941), who found the substance in the mesenchymal anlagen of the leg and tail in the mouse embryo; the present results suggest that phosphatase may be an invariable concomitant of primitive tissue. It seems not unlikely that the function of this phosphatase may in part at least be concerned in cell division. In the chick embryo both alkaline and acid phosphatase are active in almost every tissue during the stage of intense cell proliferation; but there are exceptions. The extra-embryonic cell layers are very poor in both enzymes at the stage when they are expanding rapidly over the yolk, and the myocardium is all but devoid of phosphatase during a long period of rapid nuclear proliferation. Similarly, other workers have found that active tumors do not necessarily contain much phosphatase (Gomori, 1941b; Kabat and Furth, 1941). The traces of the enzymes that persist in the heart nuclei may of course be sufficient for the mitotic needs of the tissue. Willmer (1942) has indeed shown that in cells of chick heart tissue grown *in vitro,* alkaline phosphatase activity is intensified during mitotic activity and then regresses, leaving the cell negative except for reaction in the nucleolus and centrosphere. But if the very small amount of phosphatase demonstrated by Willmer, or observed in the sectioned heart muscle, is the actual requirement of mitotic activity, then a question remains: what is the function of the excess of phosphatase which the embryo possesses above this basic minimum? In the central nervous system, for example, phosphatase is not limited to the ependyma, where proliferation occurs; and even the undifferentiated endoderm, which is relatively weak in alkaline phosphatase, has a modest amount of the enzyme in its cytoplasm, and considerably more in the nuclei.

But proliferation is only one aspect of the function of primordial cells; equally important is the preparation for the histogenetic expression of differentiation. Experimental embryology has shown repeatedly that differentiation proceeds during a long period before its effects are fully realized, for there are a great number of cases in which transplants made at intervals from regions of apparent undifferentiation display increasing ability to manifest their normal prospective value; this is of course the phenomenon which Huxley and de Beer (1934) have termed "progressive chemo-differentiation." What then of the fact that all tissues of the early chick embryo contain substantial amounts of phosphatase during their period of chemo-differentiation leading to the assumption of definite form? It may not be without

significance that phosphatase is abundant in the primitive streak and its associated structures, and appears in the true embryonic structures as soon as they are laid down. Thereafter no tissue becomes nearly free of phosphatase until its definitive form is clearly indicated; this is equally true of cardiac muscle, which is almost free of the enzyme at the time it starts to beat; of the alimentary tract, parts of which become quite free of phosphatase as they become morphologically demarcated; of parts of the brain, of cartilage, etc. Moreover the increase of phosphatase which the hinder parts of the body undergo through the second day, when chemo-differentiation is proceeding actively, is suggestive in this regard, and so is the heightened phosphatase content of the liver diverticula between their appearance and the development of true liver tissue. The relatively high concentrations of acid phosphatase in the nervous ependyma, and the concurrent proximo-distal courses of histological differentiation and phosphatase decline in the developing retina, also seem pertinent. It is interesting, too, that Krugelis (1942), working with mouse testes, observed that the high concentration of alkaline phosphatase in the spermatogonia and spermatocytes declines to negativity as differentiation proceeds. For the time being, one can merely point out these suggestive parallels; but studies now contemplated will examine in more detail the possible interdependences between the progress of developmental patterns and the changes of phosphatase content.

The basic chemical role of phosphatase in development is even more obscure. Yet considering the tremendous importance which current research is attaching to phosphate transfer, it may not be extravagant to suggest that, among the diverse chemical mechanisms that lead to diverse histologies, there is during a considerable portion of primary differentiation an invariable factor involving phosphorylation and dephosphorylation, and so necessitating the presence of phosphatases. Perhaps the synthesis of proteins under the influence of phosphate-bearing nucleic acids involves phosphatase activity; it will be remembered that Caspersson and Thorell (1941) found especially high concentrations of nucleic acid in the cytoplasm of chick embryo cells in the earliest stages of development. This view of course would relate phosphatase activity to form change, rather than to histogenetic differentiation. In any case it will be interesting to learn why two phosphatases are simultaneously present in primitive tissues, and why their relative activities in different regions vary to some extent.

Although the chemical tests have shown no differences between phosphatases in differentiated tissues and in still undifferentiated anlagen, the phosphatases which accumulate in differentiated structures should probably be regarded as existing in a different phase from the primitive enzymes. The former, that is to say, are not merely remnants preserved from the early period, but are in themselves consequent on differentiation. A comparison of activities will illustrate this point. In the case of the alkaline phosphatase, the perichondrium, epithelia of heart and liver, brush borders of secretory tubules, and several other tissues show a level of activity higher than ever attained by the "unspecialized" enzyme of pre-differentiated tissues, and the same is true, in the case of acid phosphatase, of the Wolffian duct, liver cords, metanephric tubules, and so on. In all these tissues histological differentiation seems to entail somewhere in its course a chemical differentiation in the sense of production of phosphatase to be used in the incipient functioning of the organ. Thus the mesonephros, which is functioning already on the fifth day (Boyden,

1924), assumes by that time a phosphatase distribution virtually identical with that which has been described, especially in the case of alkaline phosphatase, for the adult mammalian kidney (Gomori, 1941a, b; Kabat and Furth, 1941). But the alimentary tract, non-functional in the embryonic period, loses much of its primitive epithelial alkaline phosphatase after becoming well-launched on its course of definitive differentiation, and it does not accumulate more up to the eighth day, even though in adult chickens, as well as mammals, parts of the digestive mucosa are extremely rich in the enzyme (cf. Folley and Kay, 1936; Gomori, 1941a; Kabat and Furth, 1941). And again, in organs which do not have one or both enzymes in their adult condition (e.g., the myocardium, or the liver cells in respect to alkaline phosphatase) there is usually a swift disappearance of the enzyme after the first stages of differentiation are completed; though of course it is not contradictory that a few rather well-differentiated organs (e.g., the brain, which is poor in phosphatase in its adult condition) contain phosphatase which has no obvious functional role; in such cases the enzyme may play a part in the more advanced stages of differentiation. Thus the specific organ phosphatases accumulating as differentiation proceeds are not necessarily conterminous in origin with the primitive phosphatases of the first days, and it will probably be profitable to consider them as separate entities.

This concept of a diphasic phosphatase occurrence in young embryos agrees well with the results of Lipmann (1936), who found a peak of alkaline phosphatase content in extracts of whole chick embryos on the sixth day. The histochemical preparations certainly give the impression that there is such a peak between the fifth and seventh days, for at that time the specific organ phosphatases are accumulating rapidly while the phosphatase in undifferentiated tissues, especially mesoderm, is still present in great quantities, although it disappears very soon thereafter with the appearance of cartilage and skeletal muscle.

Among the separate observations reported, those bearing on the relation of phosphatase to ossification deserve to be commented on. Robison's hypothesis that the activity of alkaline phosphatase is a causative factor in the deposition of bone salts is now generally accepted, and my results on the long bones are in full agreement with it. These results demonstrate, as Fell and Robison (1929) have already shown for chick femora cultivated *in vitro*, that the extremely high phosphatase concentration which is associated with ossification begins to accumulate with the differentiation of hypertrophic cartilage, and that the phosphatase concentration from seven to ten days is largely confined to the perichondrium, where it is limited to the diaphysial region between the zones of flattened cells—the exact region in which bone is seen to be deposited. Further, it is clear that in all bones of the hind limb the deposition of calcium phosphate is preceded by the development of a broad zone of high phosphatase activity. This phosphatase seems to be derived from the perichondral osteoblasts in which it appears. It is true that the body of the cartilage contains reactive cells, but the low activity of the matrix indicates that these reactive cells do not, within the period of observation, eject their phosphatase, as Bourne (1943) showed that similar cells do in cartilage formed in holes drilled in adult bone. Rather the resemblance between these positive cells of the long bones and the phosphatase-rich endochondral osteoblasts of the future

vertebral bodies suggests that the former are also of endochondral function, and are not concerned in the production of perichondral phosphatase.

The data as presented in Table III demonstrate that the state of differentiation of the cartilage, the quantity of phosphatase accumulated, and the amount of calcium phosphate deposited, are interrelated. In every bone examined, it was found that the concentration of phosphatase rises at the same rate as the cartilage hypertrophies, and that when the phosphatase-rich osteoid reaches a certain thickness, bone begins to be laid down; this is equally true in the tibia, where the entire process is extremely rapid, and in the proximal phalanges, which remain for two days in the protochondrial stage, and do not commence ossification until the eleventh day. In no case was any one condition found to be out of phase with the others. Fell and Robison (1934), investigating Robison and Rosenheim's (1934) postulated second mechanism of ossification, found that this mechanism, the ability of a tissue to deposit bone salts from a supersaturated solution, develops gradually during the period when bone is normally deposited. Evidently this development is paralleled by the accumulation of perichondral phosphatase.

Recently Horowitz (1942), using the histochemical technique, has shown that in fetal rat heads of thirteen days or more glycogen and phosphatase are simultaneously present only in tissues that ossify, and he has thus given fresh impetus to the view, originally suggested by Harris (1932), that glycogen plays a role in calcification, being possibly the source of the required phosphoric esters. Considering Glock's (1940) report that in rat bones taken a few days before birth glycogen is most concentrated at the primary center of ossification, it might be of interest to know whether in the very early material investigated in this study the accumulation of glycogen also parallels the cartilage-phosphatase-bone correlation which has been observed. It is worth noting that, according to Dalton (1937), glycogen first appears in the chick liver on the seventh day, increases slightly, and then decreases between the ninth and thirteenth days, the very period in which ossification is proceeding most actively. Moreover, alkaline phosphatase vanishes from the liver cells just before glycogen begins to be stored there.

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Summary

1. Both acid and alkaline phosphatases are present in the unincubated blastoderm of the hen's egg, and in all embryonic tissues during the first two or three days of development. The concentration of alkaline phosphatase is much greater than that of acid phosphatase.

2. Phosphatase persists as long as a tissue remains undifferentiated. As differentiation proceeds, phosphatase in some cases disappears and in others accumulates in higher concentration than in the primitive phase. Alkaline phosphatase is more widespread than acid.

3. The changes in phosphatase distribution in the principal soft organs up to the eighth day, and the relation of alkaline phosphatase to bone deposition in the hind limb up to the eleventh day, are considered.

4. The effects of a variety of chemical agents on both phosphatases are reported.

5. The possible significance of phosphatases in the processes of embryogenesis is discussed.

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